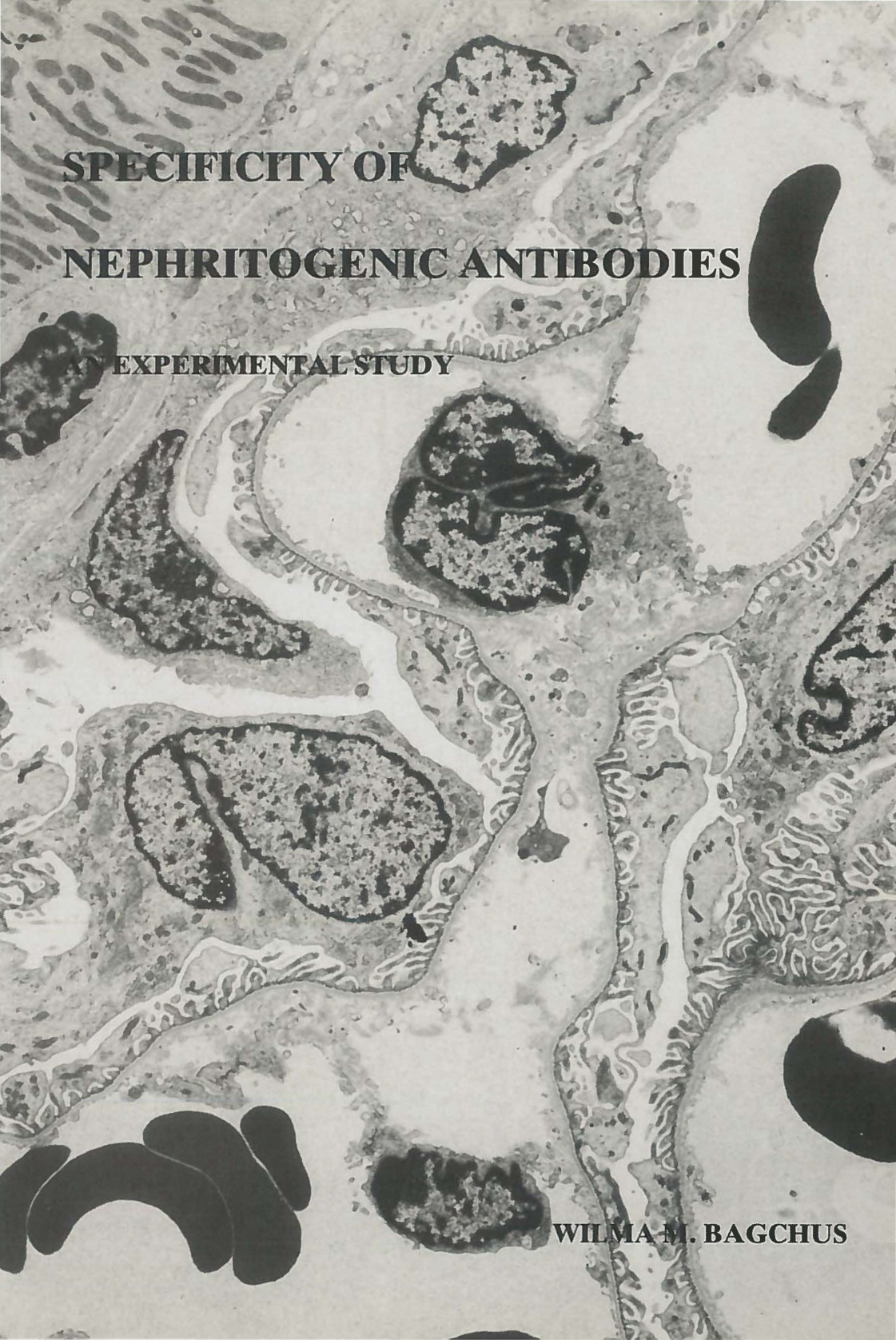
Specificity of nephritogenic antibodies : an experimental study

Bagchus, Wilhelmina Maria



SPECIFICITY OF NEPHRITOGENIC ANTIBODIES

AN EXPERIMENTAL STUDY

WILMA M. BAGCHUS

SPECIFICITY OF NEPHRITOGENIC ANTIBODIES
AN EXPERIMENTAL STUDY

STELLINGEN

I.

Immuno-electronenmicroscopie mbv peroxidase is een onbetrouwbare techniek.

II.

De rat is geen geschikt proefdier voor het bestuderen van de pathogenese van de humane idiopathische membraneuze glomerulopathie.

III.

De eigen identiteit van een wetenschappelijk tijdschrift dient niet te blijken uit de manier waarop de literatuurreferenties vermeld worden.

IV.

Het doorberekenen aan vakgroepen van overheadkosten voor derde geldstroom subsidies getuigt niet van een wetenschappelijk stimulerings beleid.

V.

Het woord personal in Personal Computer slaat niet op een persoonsvriendelijke benadering.

VI.

Het doen van uitspraken op taalkundig gebied alleen gebaseerd op het beheersen van twee talen, is even zinvol als het doen van uitspraken op nefrologisch gebied alleen gebaseerd op het bezitten van twee nieren.

VII.

De praktijk van het huidige vervolgingsbeleid van de "kleine" misdaad, heeft mede tot gevolg dat het vertrouwen van de slachtoffers in de rechtsstaat kleiner wordt.

VIII.

Hoewel een pluriforme samenleving ruimte geeft aan fundamentalistische groepen, is dit omgekeerd niet het geval.

IX.

Of Nederland een "kikkerland" is, hangt mede af van de milieubewustheid van de bevolking.

X.

Anthony's uitspraak in Shakespeare's Julius Caesar: "Friends, Romans, countrymen, lend me your ears" is overdrachtelijk bedoeld.

XI.

Wordsworth's uitspraak dat poëzie "takes its origin from emotions recollected in tranquillity" is tevens een goed uitgangspunt bij het schrijven van een wetenschappelijk artikel.

XII.

Het woord minimal in "Minimal Music" slaat niet op de waardering of grootte van het publiek.

XIII.

Hoe men graffiti ook uitspreekt, het blijft even lelijk.

Stellingen
behorend bij het proefschrift van
W.M. Bagchus
Specificity of nephritogenic antibodies
An experimental study
Groningen, 15 Oktober 1986

RIJKSUNIVERSITEIT TE GRONINGEN

SPECIFICITY OF NEPHRITOGENIC ANTIBODIES

AN EXPERIMENTAL STUDY

PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de Rector Magnificus Dr. E. Bleumink
in het openbaar te verdedigen op
woensdag 15 oktober 1986 des namiddags te 4.00 uur
door

WILHELMINA MARIA BAGCHUS

geboren te Losser

1986
DRUKKERIJ VAN DENDEREN B.V.
GRONINGEN

Promotores: Prof. Dr. Ph. J. Hoedemaeker
Prof. Dr. J. D. Elema
Referent : Dr. W. W. Bakker

To
Robert

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.

This study was performed at the Department of Pathology (Prof. Dr. Ph.J. Hoedemaeker, Prof. Dr. J.D. Elema) of the State University of Groningen.
Although this thesis bears only the name of one author, it could not have been written without the fervent support of many.
In the first place I would like to thank my parents for giving me access to the type of education necessary for the present work.
I am much indebted to Prof. Ph.J. Hoedemaeker and Dr. W.W. Bakker for their critical and stimulating guidance.
I am grateful to Prof. Dr. J.D. Elema, Prof. Dr. S. Poppema and Dr. J.J. Grond for their help, of which the screening of many kidney and lymphoid sections is only one example.
Excellent laboratory assistance was offered by Mrs Dicky van de Top, Mrs Janny Scholma, Mrs Klaske ten Hoor, Mr Klaas Kooistra, Mrs Freke Dijkhuis and other members of the "koffieclub".
I especially want to thank Mr Hans Vos whose contributions were invaluable. I greatly appreciate the electronmicroscopy work carried out by Mr Jan Donga in the Department of Medical Electron Microscopy (Prof. Dr. I. Molenaar).
Mr Harry Ferwerda and Mr Anno van Veenen took good care of the experimental animals and assisted me in many experiments.
Mr Be Siekman performed accurate technical assistance with the staining of innumerable kidney sections.
For the many drawings, slides, posters and photographs I thank Mr Hilbrand Wierenga, Mr Dick Huizinga, Mr Hemmo Nijborg, Mr Jacques Duitsch, Mr Theo Deddens, Mr Maurits Huystee and the staff of the Department of Medical Photography.
The substantial amount of last minute typework was carried out by Mrs Anneke Boer, Ms Truus Bartels-Struik and Mrs Jose Kop.
I thank Dr. J. Koudstaal for introducing me to the wonders of the PC and Mr. H.F. Vogt for laser-printing this thesis.
Thanks are due to the staff of the Department of Histology (Prof. Dr. P. Niewenhuis) who helped with various experiments (especially Dr. Davine Opstelten, Mrs Auk Wuubena, Mr Gert-Jan Deenen, Mr Boelo Meedendorp and Frans Kroese.)
Dr. J. Rozing kindly supported the monoclonal anti-Thy 1.1 antibodies.
I acknowledge the hospitality of Prof. Dr. J.F.G. Slegers of the Department of Physiology and the expert assistance of Mrs Mieke Moons in performing the perfusion studies.
I want to emphasize that besides the help mentioned above many offered moral support and friendship as well.
This study was financed by the Dutch Kidney Foundation.

INTRODUCTION

In the last decades our insight and understanding of the function of the immune system has been greatly enlarged , although new discoveries led us to believe that it is still far from complete. Simultaneously, the role of immune mechanisms in the pathogenesis of various diseases, in particular glomerulonephritis, was convincingly demonstrated in humans as well as in experimental models. Some of these models have assumed counterparts in human pathology; of others the counterpart is not well defined. However, all models contributed to our current knowledge of factors important in the pathogenesis such as immune complex deposition, formation of immune complexes in situ, complement activation and increased glomerular permeability. The focus of attention has mainly been on the mechanism of immune complex formation and the hazards of immune complexes either deposited in the glomerulus or formed in situ.

Antibodies and antigens may combine to form immune complexes in the circulation. The size of these complexes varies greatly and is associated with the antigen/antibody ratio. Although the majority of complexes are cleared by the Reticulo Endothelial System (RES), small complexes are removed slowly, remain longer in the circulation and may deposit in vessel walls and along the glomerular capillary wall (GCW). Immune complexes may also form locally in the glomerular capillary wall. This can occur when antibodies bind to antigens which are already present in the GCW, either as intrinsic GCW constituents or as antigens which are planted there from the circulation.

In this thesis two models of glomerulonephritis are studied which morphologically and clinically resemble human membranous glomerulopathy.

-Autologous Immune Complex Nephritis (AICN)

-Heterologous Immune Complex Nephritis (HICN)

In both models antibodies directed to antigens present in renal epithelial cells result in in situ formation of immune complexes in the rat kidney.

The AICN is an experimental model which was first described by Heymann et al. in 1959 [89]. They succeeded in eliciting an experimental glomerulonephritis in rats by immunizing the animals with homologous rat kidney cortex homogenate. In glomeruli of the immunized animals, rat IgG was observed in a granular pattern along the GCW. In the circulation autoantibodies were present that were directed to kidney tissue. Later these autoantibodies were shown to be directed to antigens present in the brushborder of the renal tubular epithelium. In 1969 Edgington [66] confirmed the true auto-immune nature of the immune response and renamed the disease autologous immune complex nephritis (AICN).

Various antigenic fractions have been reported to induce AICN such as a crude fraction derived from the proximal tubule called Fx1A [3, 25, 42, 52, 77,150], a more purified fraction called RTE α5 [63], mitochondria [21, 22] or pronase digested kidney cortex homogenate [127]. Recently Kerjaschki et al. reported the purification of the antigen responsible for the induction of AICN and HICN [105, 106]. The antigen is a glycoprotein of 330 kD , present in the brushborder of the proximal tubules and on the cell membrane of glomerular epithelial cells and other epithelia [59].

Auto-antibodies directed to the responsible antigen are present in the circulation of rats with AICN. After an initial rise, reaching peak levels at week 5 to 9, the antibodies decrease [70]. This is probably due to a (down)regulation of the immune response against autoantigens. Whether GP 330 is the only nephritogenic antigen involved in Heymann nephritis is uncertain, especially since other antigens present on both brushborder and rat glomerular epithelial cells (GP 90) have been claimed to be involved in the induction of the disease [7, 43, 44, 139]. AICN can be elicited in various rat strains. The susceptibility of rats for this nephritis varies, probably determined by genetic differences [160].

In the early seventies a variant of the AICN was described by several authors [21, 22, 67, 90]. In this model injection of rabbit anti-Fx1A antibodies results in granular immune complex deposits in the rat kidney immediately following the injection. In subsequent days the amount of deposits gradually increases, followed at day 7 by

the deposition of rat antibodies against the rabbit antibodies (autologous phase). Ex vivo perfusion experiments using rabbit-anti-Fx1A antibodies [57] or sheep anti-Fx1A antibody [54] showed that these immune complexes are formed by direct binding of antibodies to an antigen present in the glomerular capillary wall.

Hess [88], and later Harmon [82] and Litwin [111, 112, 113], reported that cellular immunity probably is involved in AICN. By means of the delayed hypersensitivity skin test Litwin showed cellular immunity specifically directed against Fx1A [111]. This test became positive at the onset of proteinuria [112]. Also in vitro, using the lymphocyte transformation test, cells were shown to proliferate following challenge with Fx1A [111, 112]. Tolerance against Fx1A can be induced by pretreatment of rats with Fx1A in incomplete Freunds adjuvant [82, 113]. This procedure results in the induction of lymphoid suppressor cells able to transfer unresponsiveness for AICN to naive animals. Harmon [82] showed that these suppressor cells did not inhibit sensitization to Fx1A, but modified the antibody response specifically. Recent experiments from de Heer [84] and Cheng [44] indicated that these suppressor cells reside in the thymus and, to a lesser extent, in the spleen.

Own studies on cellular immunity in AICN and HICN.

In vitro tests measuring cellular immunity in AICN [16] and HICN [17] have been performed in our laboratory by means of the migration inhibition factor (MIF) assay. It was shown that thymocytes of rats with AICN respond upon challenge with Fx1A, in contrast to control thymocytes. These results indicate the presence of thymocytes with receptors able to recognize the Fx1A antigen. Moreover, it was noticed that apart from thymocytes sensitized towards Fx1A also thymocytes were found which appeared to be sensitized against anti-Fx1A antibody, but not against control rabbit antibodies. (Fig. 1 presents a schematic picture of Fx1A and anti-Fx1A IgG). Surprisingly enough apart from rats with AICN these cells could also be found in normal rats although to a lesser extent.
From these data the hypothesis was formulated that in rats thymocytes are present that recognize anti-Fx1A in order to (down)regulate Fx1A

recognizing cells. When animals are immunized, cells with receptors for FxlA develop, leading to an increase in the number of regulatory cells, i.e. cells with the internal image of the antigen, thus mediating a suppressive effect on the number of FxlA recognizing cells.

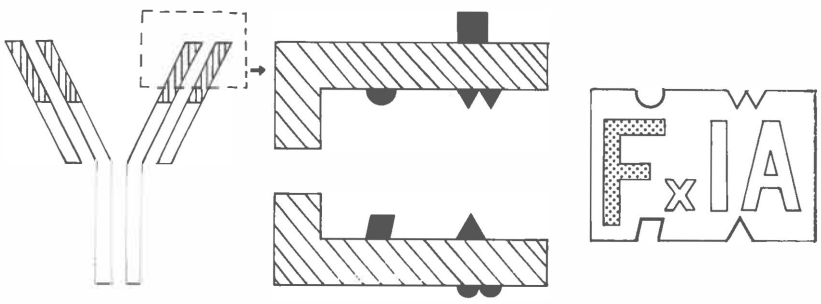


Fig.1: FxlA, anti-FxlA antibody. Figure 1 presents a schematic picture of immunoglobulins consisting of two light and two heavy chains (left figure). The variable regions (magnified in the middle figure) form the antigen binding site of the antibody, these are different in each antibody. This structure is referred to as the combining site or paratope of the antibody and recognizes determinants (or epitopes) of the antigen FxlA (right figure). The specific determinants of the antibody are idiotypes (black structures), some of them are found within the paratope, others are found outside it.

This theory fits in the network hypothesis formulated by Jerne in 1974 and summarized in Fig. 2 [100, 101].

The studies that led to the formulation of the hypothesis as well as those performed to prove it, and leading to the discovery of a new mesangial type of glomerulonephritis, constitute the contents of this thesis. Since FxlA is a crude mixture of antigens, anti-FxlA antibodies probably contain various specificities [18]. Therefore it was necessary to purify the antibody to prove the above hypothesis. Using immuno-fluorescence (IF) and cytotoxicity assays it became clear

that heterologous anti-Fx1A possesed at least two major specificities: one reacting with an epitope present on brushborder and on all thymocytes (later shown to be anti-GP 90), and one reacting with brushborder and less than 1% of thymocytes. Comparison of autologous antibodies with heterologous anti-Fx1A antibodies proved that the anti-T specificity was not found in autologous antibodies.

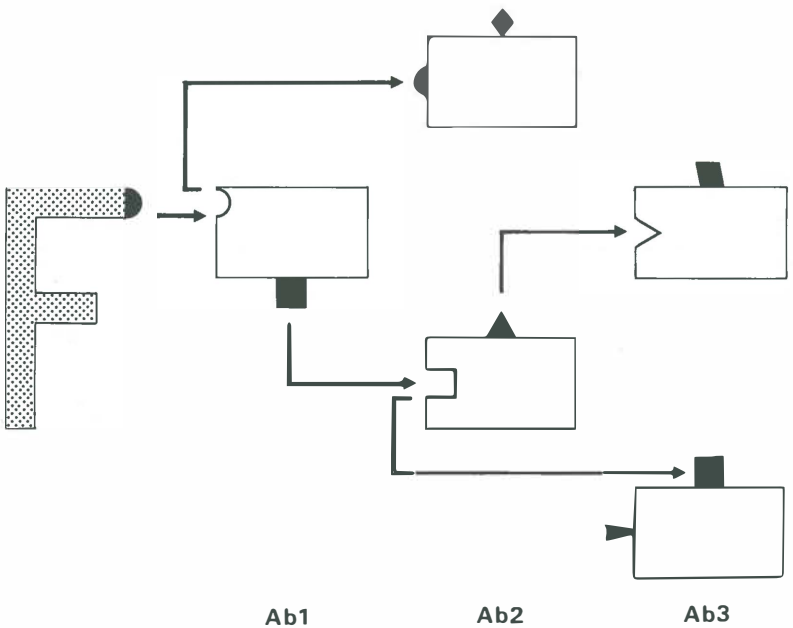


Figure 2: Network hypothesis. Antigen (Fx1A) stimulates the production of antibodies (anti-Fx1A IgG, Antibody 1) carrying a number of idiotypes. The idiotype stimulates the formation of anti-idiotypes (Ab 2) that regulate the production of the idiotype. These anti-idiotypes are in turn regulated by anti-anti-idiotypes (Ab 3), and so on. However, the immune response normally tapers off at reaching Ab 3. Among the various anti-idiotypic antibodies , in particular those with idiotypes resembling the original antigen (called internal image) are of interest for the the present hypothesis (upper Ab 2). Because anti-Fx1A is able to recognize the Fx1A antigen in the brushborder as well as a structure present on thymocytes it might be suggested that it recognizes a cell carrying a receptor bearing the internal image of the Fx1A antigen. This cell might in turn control cells able to recognize Fx1A.

When the anti-thymocyte activity was removed from the heterologous anti-Fx1A, the antibody reacted only with brushborder antigen later shown to be GP 330. Immunofluorescence on thymocytes of normal and nephritic rats indicated that the Fx1A (GP 330) bearing cell might be involved in suppression of the anti-Fx1A response, because the decrease in antibody titres coincided with an increase in the number of these cells. Since different rat strains have different susceptibilities to the induction of AIC, we were interested whether this susceptibility could be correlated to the number of anti-Fx1A (GP 330) bearing cells.

In chapter 2 the number of GP 330 bearing thymocytes in nephritic and control animals and in different (non)-responder rat strains are compared by IF techniques.
Chapter 3 reports the dual specificity observed in anti-Fx1A IgG and anti-thymocyte antibodies using cytotoxicity and MIF assays.
Chapter 4 reports the observation of anti-thymocyte activity in glomerular eluates from HICN rats. The importance of this specificity for the binding of anti-Fx1A IgG in vitro and after perfusion ex vivo is discussed. The immunoblotting experiments show that this anti-thymocyte specificity is directed against GP 90.
In chapter 5 immunohistological staining patterns on rat lymphoid organs of the different specificities in anti-Fx1A antibodies were compared with monoclonal antibodies directed to GP 330 and GP 90 epitopes. It is shown that anti-thymocyte specificity and anti-GP 90 Moab recognize T-lymphocytes in an identical pattern.
The localization of the different specificities of anti-Fx1A IgG were also studied in controlled ex vivo perfusion and after iv injection. The results are presented in chapter 6 and indicate that in vivo only anti-GP 330 antibodies play a role in immune complex formation in the GBM.
The nephritogenicity of heterologous anti-thymocyte antibodies is presented in chapter 7. The mesangial localization of these antibodies (quite distinct from anti-Fx1a IgG patterns) indicate involvement of anti-Thy 1 antibodies.
Therefore in vitro binding to the rat kidney of three different anti-Thy 1 Moabs was studied using IF and IEM techniques. The data

presented in chapter 8 show the Thy 1 antigen to be present not only on the rat mesangium but also on the GBM.
Chapter 9 describes that in vivo administration of anti-Thy 1 Moabs induces a fulminant but transient nephritis only if complement activating subclasses of these Moabs are used.
The histological and ultrastructural data presented in chapter 10 indicate that lysis of the mesangial cell is the underlying pathogenic mechanism of this unique model of glomerulonephritis.

IMMUNOREGULATION IN AUTOLOGOUS IMMUNE COMPLEX GLOMERULOPATHY
-The relation between tolerance and GP 330 bearing thymocytes.

Bagchus WM, Hoedemaeker PhJ (1), Vos JTWM and WW Bakker.

Department of Pathology, State University of Groningen
Groningen, The Netherlands.
(1) Department of Pathology, State University of Leiden,
The Netherlands.

SUMMARY

Autologous immune complex glomerulopathy (AICN) is induced by immunizing rats with a crude brushborder fraction of rat kidney tubules (Fx1A) or with purified Fx1A antigens (GP 330). In these animals anti-brushborder antibodies develop, leading to subepithelial immune complexes along the glomerular capillary wall. In rats with AICN thymocytes sensitized against Fx1A as well as thymocytes recognizing anti-Fx1A are detected. These latter thymocytes might play a role in the tolerance against the pathogenic antigen GP 330. Immunofluorescence studies were performed in which the number of anti-GP 330 binding cells were assessed in thymus cell suspensions of rats with AICN, control rats and naive rats. It is shown that increased numbers of anti-GP 330 binding thymocytes in rats with AICN are associated with a decline in the serum anti-brushborder titre. Furthermore, it is shown that the number of anti-GP 330 binding thymocytes in naive rats of the non-responder Brown Norway strain is significantly higher compared to the PVG/c and Lewis strain, which are susceptible for the induction of AICN. The correlation between the number of GP 330 bearing thymocytes and the susceptibility for AICN, indicates a role for these cells in maintaining the tolerance against the Fx1A (GP 330) antigen.

INTRODUCTION

Autologous immune complex glomerulopathy (AICN) in the rat is an experimental disease resembling human membranous glomerulopathy [63, 89]. AICN is induced by immunizing susceptible rat strains with a brushborder fraction of rat kidney tubules (Fx1A) in complete Freund's adjuvant (CFA) [69, 70, 77] or with purified FX1A antigens ie RTEα5 [63] and GP 330 [105].

Immunized animals develop circulating antibodies (Abs) against the renal tubular auto-antigen, leading to subepithelial immune complex formation along the glomerular capillary wall and resulting in proteinuria [63, 70]. The anti-Fx1A titre of the immunized animals reaches its maximum after 5 weeks, followed by a gradual decline in the following weeks [70].

In AICN rats thymocytes sensitized towards Fx1A can be detected using the migration inhibition test (MIT) [17, 18]. In addition, thymocytes sensitized towards anti-Fx1A IgG are present in these rats, and to a lesser extent also in naive animals.

From these data it was hypothesized that thymocytes bearing these receptors might play an active role in the maintenance or loss of tolerance against Fx1A and consequently in the susceptibility for the induction of AICN in these animals. Such a regulation could be exerted by anti-idiotypic antibodies [133] or by cells , expressing idiotypes which are 'internal images' of the original antigen [100, 101].

To test this hypothesis we studied the presence of GP 330 epitopes on thymocytes of naive rats both susceptible or resistant to the induction of AICN. In addition the presence of GP 330 was studied on thymocytes from susceptible rats in which AICN had been induced as well as in thymocytes from control animals.

Antigen. Fx1A antigen was prepared from the cortices of normal Wistar rat kidneys, as described by Edgington [63] and diazotized according to the method of Gribnau [85].

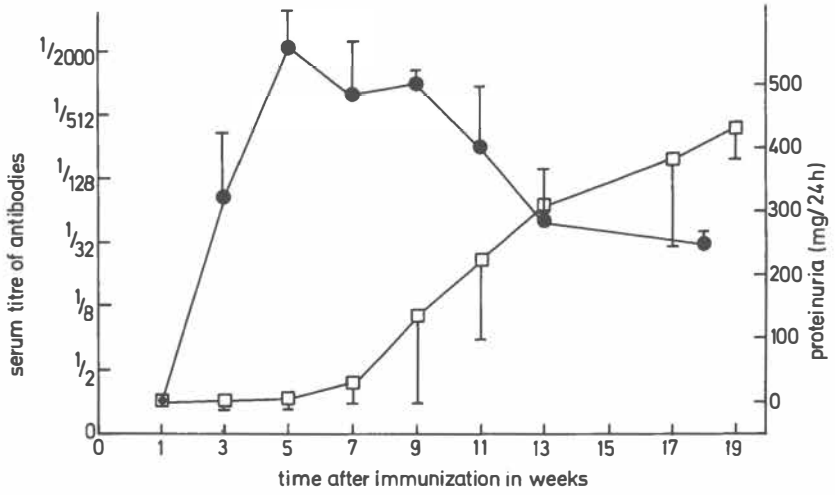


Figure 1. Proteinuria (open dots) and serum antibrushbor titre (closed dots) in PVG/c rats in which AICN was induced at the age of 8 weeks. The mean urinary protein excretion (± 1x s.d.) starts to rise after week 7 throughout the observation period. The titre of antibrushborder antibody shows a sharp rise in the first 4 weeks and subsequent gradually decline.

Mode of immunization. At the age of 8 weeks rats were immunized with 500 μl of a CFA emulsion containing 5 mg Wistar Fx1A and 1 mg Mycobacterium tuberculosis H37Ra (Difco, Detroit, USA). The inoculum was injected intramuscularly (im) and subcutanously (sc). The dorsum of the two hind footpads received 2 x 10^8 Bordetella pertussis bacteria (Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands). Figure 1 depicts the serum titre of anti-brushborder antibodies and the proteinuria observed in PVG/c rats immunized according to this protocol.

Animals. Three inbred strains were used. The PVG/c (haplotype RT1 c) and Lewis (haplotype RT1 l) were Groninger rat strains, the Brown Norway rat strain (haplotype RT1 n) was purchased from TNO, Rijswijk, The Netherlands. The animals were fed with pellet food (RMBH, Woerden, The Netherlands) and received water ad libitum.

Preparation of heterologous antibodies. Rabbit antisera against Fx1a were prepared and tested as described previously [67]. IgG was purified by ion-exchange chromatography on diethylaminomethyl cellulose (DE52, Whatman Kent, England) with 0.01 m buffered saline (pH 8.0) as elution buffer. Subsequently, this IgG was absorbed to sonicated rat glomeruli as described by Feenstra et al [67], dialysed against PBS for 72 h , adjusted to a final concentration of 10 mg/ml and stored at -20 C until use. Rabbit-anti-rat albumin IgG (Ra-Alb) was prepared and tested for specificity as described earlier. [57]

Absorption procedures. Rabbit-anti-Fx1A IgG was routinely absorbed with packed rat erythrocytes (1 ml of 100 μg IgG with 0.3 ml packed erythrocytes for 60 min at 4 C) and subsequently with washed liver tissue extract (20 mg extract per ml IgG solution, containing 100 μg IgG, for 3 h at room temperature followed by absorption overnight at 4 C) to remove non-specific 'anti-rat' antibodies. A part of the anti-Fx1A IgG was made monospecific by absorption with lyophilized rat heart tissue extract (250 mg extract/100 μg IgG) to remove anti-T cell specificity, as described earlier [14].These antibodies are shown to react with GP 330 [14] and are referred to as monospecific anti-GP 330.

Preparation of cell suspensions. Cell suspensions were prepared from fresh thymi of rats with AICN, CFA treated rats or naive rats as described earlier [19]. Briefly: thymus was taken out and kept in cold ,calcium and magnesium free, Hank's balanced salt solution (HBSS), pH 7.3. After removing the adhering lymph nodes and fat, the thymus was pressed through a 40 mesh sieve under constant pouring of chilled HBSS of 4 C. Cell suspensions were washed two times in HBSS and one time in phosphate buffered saline, containing 5% heat inactivated foetal calf serum and 20 mM NaN_3. The cells were counted

in a Coulter counter (Coulter Electronics Ltd, Harpender England) and viability was tested using 1% eosin solution.

IMMUNOFLUORESCENCE MICROSCOPY

Direct immunofluorescence. To detect immune deposits in kidneys of rats with AICN direct immunofluorescence was performed on 4 μ cryostat sections according to standard methods [70]. Sections were washed with PBS for 60 min at room temperature. For detection of rat IgG or rat C3, fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-rat IgG (RARa) or FITC-conjugated rabbit anti- rat C3 (RaC) (Nordic, Tilburg, The Netherlands) was used. After 30 min incubation with these FITC-conjugated antibodies, the kidney sections were washed with PBS, embedded in mounting medium (Gurr, pH 8.0) and examined under a Leitz fluorescence microscope with Ploemopak illumination [70].

Indirect immunofluorescence. Evaluation of individual serum anti-Fx1A titres was done by serial dilution of autologous serum in vitro incubated on normal rat kidney sections. After incubation with antiserum for 30 min at room temperature, washing in PBS and incubation with FITC-conjugated RARa for 30 min sections were processed according to standard methods. Heterologous anti-Fx1A IgG titres were assayed similarly using FITC-conjugated goat anti rabbit IgG (GAR) (Nordic, Tilburg, The Netherlands).

Indirect membrane immunofluorescence. Thymus cell suspensions of AICN, control and naive rats were stained with (heterologous) monospecific anti-GP 330 to assess the number of cells on which Fx1A (GP 330) epitopes were present. Freshly prepared thymuscells (2x 10^6) were incubated in triplo with 50 μl of either anti-Fx1A IgG-T or anti-rat albumin IgG (200 μg/ml) for 30 min at 4 C. The cells were washed twice by centrifugation (150 g for 10 min) with PBS, pH 7.3, containing 5% heat inactivated foetal calf serum (Gibco) and 20 mM NaN3 to prevent capping. Cellsuspensions were incubated with 50 μl FITC-conjugated GAR (1/60) for 30 min at 4 C and subsequently washed 3 times and resuspended in 50 μl mounting medium consisting of 7 ml glycine, 3 ml glycine-HCl (pH 8.6), 20 mM NaN3, and phenylenediamine (1mg/ml) to prevent fading of fluorescence [102]. Both the number of

fluorescent cells as well as the total number of cells of each cell suspension were counted using fluorescence microscopy (Leitz, Ploemopak illumination) or light microscopy respectively. Of every incubation at least 1000 cells were counted. In a series of 6 experiments the amount of fluorescent cells was assayed within 3000 counted cells per nephritic and per control animal. In an additional series of three experiments this was done within 21000 counted cells per animal. In experiment B, the number of fluorescent cells within 4000 cells of each naive animal were assayed.

EXPERIMENTAL DESIGN :

Experiment A. AICN was induced in 9 female PVG/c rats as described above. Equal numbers of age and sex matched control animals were immunized only with CFA and Pertussis vaccin. Fx1a (GP 330) epitopes on thymocytes of AICN and control rats were studied on thymus cell suspensions between 7 and 17 weeks after immunization using membrane IF with monospecific anti-GP 330.

Experiment B. Three female rats of the BN, Lewis and PVG/c strain were immunized with Fx1A at the age of 8 weeks as described above. Urinary protein excretion was measured every other week after induction of AICN by placing the rats in metabolic cages with free access to tap water. The amount of urinary protein was measured using the biuret method, as described previously [69]. At week 5 and week 10 blood was collected from all rats by puncture of the orbital vein. Serum titres of anti-brushborder abs were assessed by IF as described above. Unilateral nephrectomy was performed 8 weeks after induction of AICN, and the presence of immune complexes and rat complement in kidneys was evaluated by IF microscopy. The number of Fx1A (GP 330) bearing thymocytes in 3 naive rats of all strains was studied at week 8, 15 and 22. Thymuscell suspensions were prepared, and incubated with monospecific anti-GP 330 or anti-rat albumin IgG, as described above. Each incubation was done in triplo, and of each incubation 1300 cells were studied for the presence of anti-GP 330 bound cells. In addition at week 8 the experiment was repeated two times.

RESULTS

Specificity of antibodies.

Heterologous anti-Fx1A IgG decorated the brushborder of the proximal tubules as well as normal rat thymocytes membranes. Following absorption with heart tissue extract to remove anti-T cell specificity [14] staining of thymocytes was prevented (<1% normal thymus cells) while the staining of the brushborder remained present. Earlier experiments confirmed the presence of anti-GP 330 activity in these absorbed antibodies [14]. There was no staining of normal kidney sections with monospecific rabbit-anti rat albumin IgG.

EXPERIMENT A

All immunized PVG/c rats developed the typical features of AICN [70]. No antibrushborder antibody or glomerular pathology was found in the control rats. The percentages of thymocytes from nephritic and control animals able to bind monospecific anti-GP 330 are given in table 1. Between week 7 and 17 after AICN induction the number of Fx1A (GP 330) bearing thymocytes remained fairly constant, and the mean of 6 experiments was calculated.(Table 1A). In nephritic rats 0.60 % of thymocytes stain with monospecific anti-GP 330 and in control rats 0.41% of thymocytes.

Since these percentages are very low, three additional experiments were performed in which 21000 cells were counted for the number of GP 330-bearing cells (Table 1B). Thymocytes from individual rats incubated with monospecific anti-GP 330 resulted in 0.94% positive staining of cells from nephritic rats and 0.57% of thymocytes from control rats.

Incubation of thymocytes from either nephritic or control animals with rabbit-anti-rat albumin IgG did not result in positive staining of more than 0.11% of these cells (not shown in table 1).

EXPERIMENT B

Urinary protein excretion and anti-brushborder ab titre.

The mean urinary protein excretion of the three rats of the different rat strains at various times after immunization is shown in figure 2.

At week 15, mean protein excretion is around 150 mg/24 h in the PVG/c strain, around 20 mg/24 h in the Lewis strain and absent (< 7 mg/24h) in the BN strain.

Table 1 Percentage of monospecific anti-GP 330 binding cells present in thymuscell suspensions of nephritic and control rats.

Table 1 A

	nephritic rats	*control rats*
Exp 1	*0.68 ± 0.66*	*0.66 ± 0.33*
Exp 2	*0.49 ± 0.17*	*0.40 ± 0.07*
Exp 3	*0.72 ± 0.15*	*0.22 ± 0.20*
Exp 4	*0.76 ± 0.13*	*0.55 ± 0.26*
Exp 5	*0.33 ± 0.15*	*0.19 ± 0.12*
Exp 6	*0.60 ± 0.18*	*0.44 ± 0.14*
Total Mean (± 1x sd)	*0.60 ± 0.16*	*0.41 ± 0.18*

P < 0.05

Table 1B

	nephritic rats	*control rats*
Exp 1	*0.97 ± 0.17*	*0.57 ± 0.13*
Exp 2	*1.00 ± 0.26*	*0.59 ± 0.12*
Exp 3	*0.85 ± 0.18*	*0.54 ± 0.12*
Mean ± 1x sd	*0.94 ± 0.08*	*0.57 ± 0.03*

P < 0.005 .

Legends table 1: Each experiment represents the percentages of thymocytes stained with monospecific anti-GP 330 and FITC-conjugated goat-anti-rabbit IgG, from one nephritic versus one control rat. Percentages of staining cells were assessed in each incubation in triplo and their means (± standard deviation) are listed. Either 3000 cells (Table 1A) or 21000 cells (table 1B) were counted to assess the number of anti-GP 330 bearing cells. Staining with rabbit-anti-rat albumin always remained below 0.11 % positively stained cells.
(Statistical significance P< 0.05 [table 1A] or P< 0.005 [Table 1B] was determined using Students t-test).

The results of the mean titre (± 1x s.d.) of serum anti-brushborder antibodies 5 and 10 weeks after immunization of three rats of each strain are represented in figure 3.
Table 2 represents a statistical analysis of the differences in the mean anti-brushborder titres, indicating significant differences

between the PVG/c and the BN at week 5 and 10. The mean anti-brushborder antibody titre of the Lewis is intermediate between these two rat strains.

Immunofluorescence findings in rat glomeruli

All glomeruli in kidney sections obtained 8 weeks after induction of AICN from all 3 rats of the PVG/c and Lewis strain showed granular deposits of rat IgG typical of Heymann nephritis. None of the BN rats showed granular deposits along the GCW in their kidneys.

In one animal no IgG could be detected, in the other two rats some mesangial deposits could be seen. Granular deposits of rat C3 along the GCW were detected in all PVG/c rat kidneys. Kidneys of all 3 Lewis rats showed faint granular staining for C3 along the GCW. One BN rat kidney did not show any C3 staining, the other two showed minor deposits of C3 in the mesangial area.

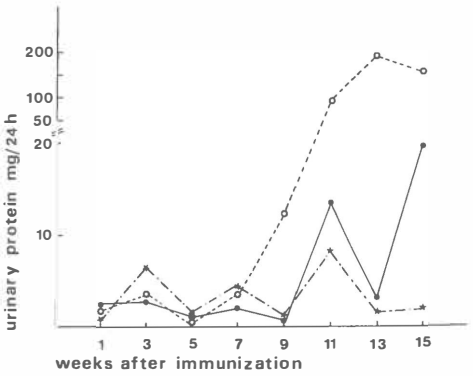


Figure 2 Protein excretion was measured biweekly in all three rats of all three strains after immunization with Fx1A. The mean urinary protein excretion is depicted in figure 2. (PVG/c open dots, Lewis black dots and Brown Norway stars).

Percentage and total number of GP-330 bearing thymocytes.

The mean (n=3) total number (± 1 x s.d.) of thymocytes present in the thymus of each rat strain at week 8, 15 and 22 is represented in figure 4. In each strain the total number of thymocytes decreases between the 8th and 22 week.

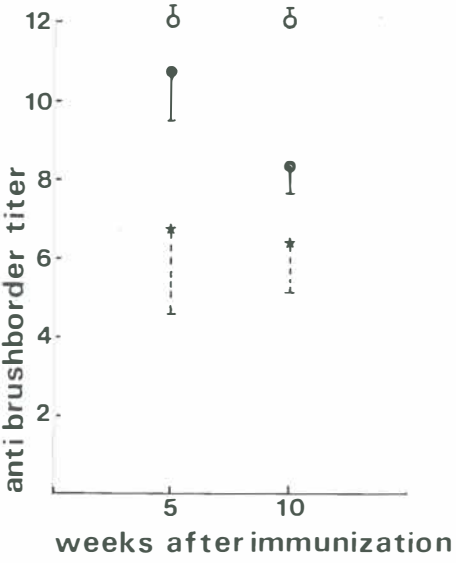


Figure 3 Three female rats of each strain at the age of 8 weeks were immunized with Fx1A. The serum antibrushborder antibody titres of the three rats were evaluated 5 and 10 weeks after immunization. The mean titre ± 1x s.d. is shown in figure 3. (PVG/c open dots, Lewis black dots ,Brown Norway stars).

The mean (n=3) percentage (± 1x s.d.) of anti-GP 330 binding cells present in thymus cell suspensions of three rat strains, at different ages is represented in Figure 5. Statistical analysis of the different percentages of anti-GP 330 binding thymocytes is depicted in table 3.

Table 2 Statistical analysis of the differences in the anti-brushborder antibody titre between the various rat strains.

	week 5				week 10		
	PVG/c	Lewis	BN		PVG/c	Lewis	BN
PVG/c	\			PVG/c	\		
Lewis	ns	\		Lewis	S4	\	
BN	S3	S2	\	BN	S4	S1	\

ns = not significant

S1 = P <0.05, S2 = P< 0.025, S3 = P< 0.01, S4 = P< 0.005 (student's T-test)

At week 15 and 22 the percentages anti-GP 330 bearing thymocytes observed in the BN rats are significantly higher ($P< 0.005$) than in the PVG/c and Lewis rats.From the data presented in figure 4 and 5 the total number of GP 330 bearing thymocytes per animal of each strain can be calculated. The mean $\pm$ 1x s.d. of each strain at week 8, 15 and 22 is presented in figure 6.

DISCUSSION

Immunization of rats with Fx1A leads to both humoral and cellular immune responses against the disease inducing antigen [17, 82, 84, 111, 112, 113]. In earlier studies we observed that thymocytes of immunized rats were able to recognize specifically heterologous anti-Fx1A IgG [17, 19]. The question was raised whether this in vitro recognition of anti-Fx1A paratopes by thymocytes , expressing receptors with "internal images" of the auto-antigen , might play a role in the maintenance or loss of tolerance against the Heymann antigen.

To investigate this hypothesis further, we induced an AICN in PVG/c rats and studied thymocytes of nephritic and control rats at various times between week 7 and 17, for the presence of anti-GP330 recognizing cells using an IF assay in vitro. In addition normal rats of three susceptible and resistant rat strains were compared for the numbers of anti-GP 330 binding thymocytes present. It is shown that a 0.60% of thymocytes from nephritic rats bind with rabbit anti-GP 330 IgG , compared to 0.41% of thymocytes from control animals (table 1A). This difference in the relative amounts of anti-Fx1A binding cells in nephritic rats compared to control is statistically significant ($P< 0.05$). To exclude sampling errors we added some experiments in which 21.000 cells per individual incubation were evaluated for anti-GP 330 binding capacity (table 1B). Although the percentages positive cells counted in the latter series of experiments are higher in both nephritic and control animals, again a comparable statistically significant difference ($P< 0.005$) could be shown between cells from diseased versus control animals (fig 1B). It is likely therefore that

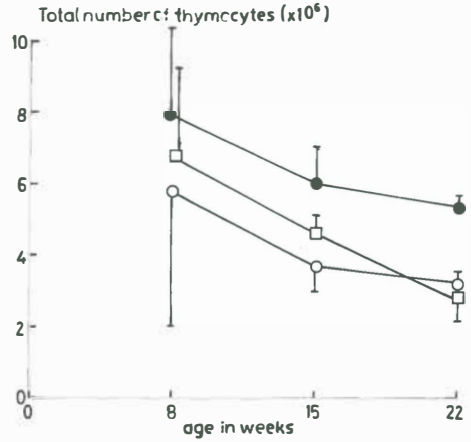


Figure 4 The mean ± 1x s.d. of the number of cells present in the thymus of three rats of the Lewis (black dots) , PVG/c (open dots), or Brown Norway strain (open squares) at the age of 8, 15 and 22 weeks respectively is depicted in figure 4.

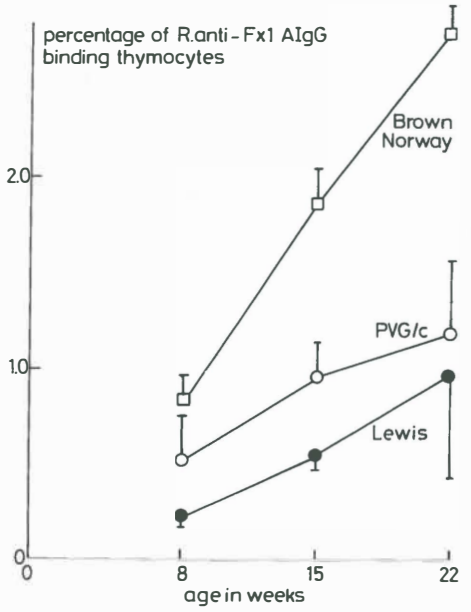


Figure 5 The mean percentage (± 1x s.d.) of GP 330 bearing thymocytes present in three rats of the Lewis (black dots), PVG/c (open dots) and Brown Norway strain (open squares) at the age of 8,15 and 22 weeks is depicted in figure 5.

relative more thymocytes of nephritic rats as compared to control rats bear membrane structures binding paratopes of rabbit anti-Fx1A antibodies.

Table 3 Statistical analysis of the differences in the percentage of anti-GP 330 binding thymocytes between the various rat strains.

	week 8			week 15			week 22		
	P	L	BN	P	L	BN	P	L	BN
P	\			P \			P \		
L	ns	\		L ns	\		L ns	\	
BN	ns	S	\	BN S	S	\	BN S	S	\

ns = not significant
S = P< 0.005 (students's T-test)

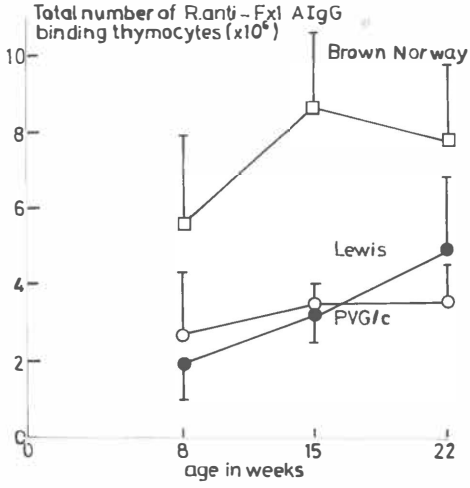


Figure 6 The percentage of GP 330 bearing thymocytes in each animal was multiplied with the total number of thymocytes present in each animal, indicating the total number of GP 330 bearing thymocytes present in each animal. The mean (± 1x s.d.) of the total number of GP 330 bearing thymocytes in three rats of each strain at the age of 8,15 and 22 weeks is depicted in figure 6. (Lewis, black dots, PVG/c open dots and Brown Norway open squares.).

In addition to these results it was shown that the number of GP 330 bearing thymocytes in the non-responder Brown Norway rat strain is significantly higher as compared to the PVG/c and Lewis strain, which are relatively susceptible for AICN [70, 84, 159, 160]. This finding again suggests a role for these cells in maintaining the tolerance against the Fx1A antigen.

As can be seen from fig 3, anti-BB titres in the BN rats were lower than in the Lewis rats and significantly less than in the PVG/c rats. In addition, the rats of the PVG/c strain developed high proteinuria, rats of the Lewis strain developed moderate proteinuria , and the BN strain rats did not develop proteinuria. This is in accordance with IF findings in the glomeruli of these rats, showing membranous deposits of rat IgG and C3 in the PVG/c rat, rat IgG and some C3 deposits in the Lewis rat also in a granular fashion and no deposits of rat IgG or C3 along the GCW in the Brown Norway rat. The present data suggest that the BN rat strain is relatively resistant for AICN, as compared with the PVG/c and Lewis strain, which is in line with findings of other authors [45, 159, 160].

The nature of the thymocyte receptor was studied using heterologous anti-T depleted anti-Fx1A antibody for technical reasons, because preliminary studies had shown that indirect IF with autologous anti-Fx1A resulted in undesirable high background staining. The exact nature of the thymocyte membrane component recognized by heterologous anti-Fx1A is unknown. The data from the immunoblotting analysis however show that anti-T depleted anti-Fx1A used for membrane fluorescence is directed to a GP 330 kD epitope. Since GP 330 is claimed to be the nephritogenic Heymann antigen [105], it is suggested that the nature of the thymocyte receptor is similar (if not identical) to the Heymann antigen.

Two explanations for the presence of such a thymocyte membrane component emerge. Firstly Fx1A might have been picked up from the circulation in diseased rats and to a lesser extent in control and normal animals and passed over to thymocytes. Secondly nephritic rats bear a Fx1A-like (GP 330) receptor structure on a subset of their thymocytes, which is also present although to a lesser extent in untreated rats.

The first possibility is unlikely for two reasons. Antigen passing over from macrophages to thymocytes is unlikely [171] and the presence of Fx1A in the circulation of normal and nephritic rats is controversial [128]. The second possibility proposing a subset of thymocytes with an antigen mimicking receptor seems more likely for several reasons. Anti-idiotypic antibodies with internal images of the original antigen have been described [47, 68, 134, 154, 172].

Suppressor T-cells might express receptors bearing idiotypic and anti-idiotypic determinants on their cell membrane [29, 30, 132, 162, 180]. Furthermore, such T-cells were shown to regulate the immune response of other T or B cell clones expressing distinct idiotypes [162, 168]. Although a possible idiotypic receptor nature of an Fx1A-like structure on thymocytes remains to be demonstrated, the relative amount of idiotype bearing cells of about 1% is in agreement with data of other authors [73, 164, 180]. The assumption of an idiotypic determinant on the T-cell which can bind to anti-self receptors fits also in the idea of a suppressive role for these cells in regulation of anti-self immunity. Antigen specific receptors of T-suppressor or suppressor precursor cells, expressing idiotypic determinants are described in other experimental systems as well [73, 162].

Whether the present GP 330 bearing thymocyte is a real suppressor cell, remains to be elucidated. Since the percentages of these cells detected in our rats are extremely small, functional studies to elucidate this question will be difficult. The presence of suppressor cells in the thymus has been reported in several auto-immune models in the mouse [129, 145] and rat [26, 125], as well as in the thymus of mice with neonatally induced tolerance to bovine serum albumin [32]. In AICN it has been shown that preimmunization with anti-brushborder antibodies inhibits the production of these antibodies and the development of AICN [62]. In addition, high dose tolerance to Fx1A can be induced by preimmunizing rats with Fx1A in incomplete Freund's adjuvant. Other authors [46, 84] have been able to demonstrate the presence of Fx1A specific suppressor cells in the thymus and to a lesser extent in the spleen of these tolerant animals, suggesting that resistance against AICN is mediated by antigen specific suppressor cells. The evidence of functional Fx1A specific suppressor cells present in AICN coinciding with a decline in antibody titre by de Heer is in accord with our data [85].

The increased number of GP 330 bearing thymocytes in AICN rats associated with the decrease in the antibody titres, and the correlation between the number of GP 330 bearing thymocytes and the susceptibility of the various rat strains for the induction of AICN, support the hypothesis that the GP 330 bearing thymocyte might be the antigen specific suppressor cell of AICN.

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES.

I. Evidence on anti-T-cell specificity in nephritogenic antibodies detected by cytotoxicity and MIF assays.

WW Bakker, WM Bagchus, JTWM Vos. K Kooistra and PhJ Hoedemaeker.
Department of Pathology, State University of Groningen, Groningen,
The Netherlands.

SUMMARY

Heterologous antibodies directed to brushborder antigens of rat kidney tubules are nephritogenic in that an immediate immune complex glomerulopathy occurs after injection of these antibodies into normal rats. Since previous observations suggested the presence of anti-T-cell specificity within these antibodies, we now compared the specificities of these antibodies with those of heterologous anti-rat thymocyte antibodies, using immunofluorescence (IF), cytotoxicity and migration inhibition (MIF) assays. The results suggest that anti-brushborder antiserum contains anti-thymocyte specificities, while antithymocyte antiserum contains anti-brushborder specificity. These specificities could be removed selectively from both of the antisera by absorption with insoluble tissue extracts containing the appropriate antigens, i.e. thymocyte antigens or brushborder antigens respectively, indicating that two specificities are involved rather than only cross-reacting antibodies.
Since it is unlikely for several other reasons that this feature is simply the result of an immunologic cross-reaction due to antibodies raised by immunization with purified kidney tissue antigens, this dual specificity of anti-brushborder antibody might play a role in the pathogenesis of experimental glomerulopathy.

INTRODUCTION

Although our understanding of the various forms of human immune complex glomerulonephritis is incomplete, it is generally agreed that glomerular damage may be caused by the presence of immune complexes along the glomerular capillary wall [48, 74].

Recently studies from our laboratory [57, 92] and from others [54, 130] have shown that immune complex formation in some forms of experimental immune complex glomerulonephritis in the rat may occur by in situ binding of nephritogenic antibodies to subepithelial structures of the glomerular basement membrane (GBM) rather than by deposition of circulating immune complexes.

Apart from specificities directed to tubular brushborder antigens, heterologous nephritogenic antibodies appeared to contain also specificities against rat thymocytes [18].

This observation as well as an additional finding that heterologous anti-thymocyte serum reacted also with brushborder antigens was surprising to us in that only specificity to brushborder antigens occurred and not for instance to GBM antigens, as demonstrated by IF techniques on frozen kidney sections of the rat. Moreover, since rabbit antibodies raised to other rat tissues did not show this dual specificity, we decided to study this phenomenon further. Therefore we have compared the activity of these nephritogenic anti-brushborder antibodies with heterologous anti-thymocyte antibodies in vitro, following absorption with various tissue antigens. The results suggest that heterologus anti-brushborder antibodies contain both anti-thymocyte as well as anti-brushborder specificities. From absorption studies with either brushborder or thymocyte membrane antigen containing tissue extracts it is concluded that the cytotoxic actitivity directed to thymocytes present in both of the antisera studied is due to specificities directed against thymocyte membrane antigens, while on the other hand immunofluorescence as well as migration inhibition assays following absorption with these tissue extracts suggest also the presence of specificities directed against brushborder-like structures in both, anti-brushborder as well as anti-thymocyte sera. In view of the concept of immune complex formation in experimental glomerulopathy, whereby in situ binding of anti-brushborder antibody to brushborder-type antigens occurs at the

epithelial side of the GBM [57, 92, 54, 130], it is conceibable that also anti-T-cell antibodies are involved in this immune complex formation. Such a role to play for anti-T-cell specificities may be important in the pathogenesis of heterologous immune complex glomerulonephritis in the rat.

MATERIALS AND METHODS

Animals.Female outbred Wistar rats weighing 200-300 g were used throughout the study. The rats were fed with RMHEB (Hope Farms, Woerden,The Netherlands) and received water ad libitum. For preparation of antisera against brushborder antigen, white rabbits and chinchillas (TNO, Zeist, The Netherlands) were used.

Preparation of antigens and antibodies. Soluble brushborder antigens for immunization were prepared from Wistar rat kidneys as described elsewhere [64], whereas also an insoluble brushborder fraction was prepared for absorption studies [65].
Thymocyte membrane antigen was prepared by extraction of fresh rat thymus tissue with a warring blendor in phosphate-buffered saline (PBS) at 4oC. The homogenate was washed three times with PBS and spun down (30,000 x g) and lyophilized. Rat brain tissue, which is known to contain a considerable amount of thymus specific antigens [176], from which the choroid plexus was removed, was prepared in a similar manner. Both heterologous (rabbit) anti-rat brushborder IgG as well as non-immune rabbit IgG were prepared as described elsewhere [67];heterologous anti-thymocyte antibodies were obtained from Nutacon, USA. The latter antibody showed a 50% kill at 1/128 dilution and 100% kill at 1/32 upon normal rat thymocytes using a complement-dependent cytotoxicity assay [135].
To remove non-specific antibodies, batches of heterologous antibodies were routinely absorbed to fresh (rat) erythrocytes as well as to lyophilized rat heart tissue extracts. Pilot experiments showed that equal results were obtained when either IgG fractions, γ globulin fractions or full sera were used in the test systems described; therefore we employed IgG fractions in the MIF assays,

whereas in the cytotoxic assays and in the IF assays γ globulin fractions or full sera were used.

Absorption procedures. Routine absorption of rabbit antibodies was carried out by incubating the serum or IgG fraction (100 μg IgG/ml) in tissue culture medium or Hanks' balanced salt solution (HBSS) with washed rat erythrocytes in HBSS at 4oC. After one hour the RBC were removed by centrifugation and the antibody was absorbed with lyophilized heart tissue extract. Absorptions of heterologous antibodies to lyophilized rat tissue extracts were performed at room temperature for two hours using a head-over-head rotation device (0.75 rotations per minute), whereas after this period the incubation was continued overnight at 4oC following the centrifugation 30,000 x g for 60 min to remove the tissue antigens and immune aggregates. Repetition of this procedure showed no change of antibody activity as tested using the different techniques described.

Cytotoxicity assay.A cytotoxicity test according to Okumura [135] was used.
In brief: serially diluted heat-inactivated serum samples were mixed with equal suspensions of freshly prepared thymus cells (3 x 10^6 cells/0.1 ml) in glass tubes at room temperature. Parallel series with similar dilutions of normal heat-inactivated rabbit serum were processed in the same way to evaluate background lysis. After 30 min, diluted guinea pig serum (1:10) was added in an equal volume and the plates were incubated another 60 min at 37oC. Relative cell lysis was evaluated after addition of eosin red in saline in a final concentration of 0.2%.

MIF-assays. Direct MIF assays were carried out as described elsewhere [15]. In brief: cell suspensions were prepared from fresh thymuses at 4oC and washed three times in HBSS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Difco). Cell suspensions were incubated with or without the testmaterial for 60 min at 37 C with RPMI containing 10% heat-inactivated horse serum (National Institute of Health, Zeist, The Netherlands) and penicillin and streptomycin as indicated for HBSS. After this period, suspensions were spun down (150 x g; 5 min), and supernatants were removed leaving 10% by volume in

the tube. The cells were mounted in glass capillary tubes (20 microliter Drummond) and placed into sterilized migration chambers (Sterilin) and tissue culture medium supplemented with appropriate test-material concentrations were added. After 5 and 18 hours migration areas were measured according to standard techniques. All migration inhibition assays were done in quadruplicate. The results were expressed as migration indexes calculated as a percentage of migration:

$$\frac{\text{mean migration area in the presence of antigen}}{\text{mean migration area without antigen}} \times 100\%$$

Immunofluorescence techniques.

Kidney fragments were snap-frozen in precooled Freon (-100 C) and cryostat sections were cut and prepared as described elsewhere [57]. Indirect fluorescence was performed using either rabbit anti-brushborder globulin or rabbit anti-thymocyte globulin as first incubation step and with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Nordic) as the second incubation step. Control incubations were done with normal rabbit globulin as the first incubation and processed as was described for anti-brushborder or anti-thymocyte serum.

RESULTS

Specificity of anti-thymocyte serum.

Investigation of the specificity of the commercially obtained rabbit anti-rat thymocyte serum showed that this serum was directed against normal Wistar rat thymocytes as was tested in our standard cytotoxicity assay using guinea pig complement (100% kill at 1/32 and 50% kill at 1/128 dilution), whereas 97% of the anti-thymocyte cytotoxic activity could be removed after a single absorption step using thymocyte membrane extracts.

Absorption with rat erythrocytes, heart tissue extract or liver tissue extract did not decrease the anti-thymocyte titre. About 40% of normal rat spleen cells were lysed by anti-thymocyte serum at a dilution of 1:32 using the standard cytotoxicity assay.

Fluorescence of anti-brushborder and anti-thymocyte serum.
Anti-brushborder globulin incubated with cryostat sections of rat kidney tissue stained the brushborders of the proximal tubules as can be seen from Figure 1a. After absorption of this antiserum with an insoluble brushborder antigenic fraction this staining became negative whereas absorption to thymus membrane antigens did not remove this specific binding (Table 1).
Anti-thymocyte globulin also stained the brushborder of cryostat sections from rat kidney tissue (Fig. 1b) and in this case the binding of specific antibodies to the tubular brushborders could be prevented by absorption with brushborder antigens as well as by absorption with thymus membrane antigens (Table 1).

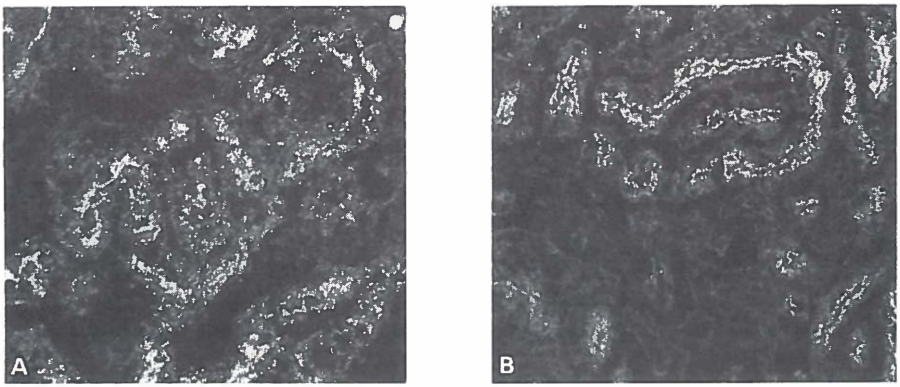


Fig. 1a and 1b. Indirect fluorescence of brushborders of cryostat sections from normal rat kidneys after incubation with anti-brushborder antibody (a), or anti-thymocyte antibody (b), following incubation with FITC-conjugated goat anti-rabbit IgG.

Cryostat kidney sections were incubated with either anti-brushborder or anti-thymocyte antibodies and FITC conjugated goat anti-rat IgG, following absorption with various tissue antigens.

Cytotoxicity.

The data obtained from cytotoxicity tests using either anti-brushborder, anti-thymocyte antibodies or normal rabbit serum are summarized in Table 2 (symbols not in parentheses).

As can be seen from this table, anti-brushborder antibody with guinea pig complement shows a clear cytotoxic response to normal rat thymocytes in vitro which can be removed by absorption to both thymocyte antigens as well as brushborder antigens. Anti-thymocyte globulins together with guinea pig complement give also rise to a considerable lysis of normal rat thymocytes which could be diminished a great deal by absorption with brushborder antigens. Absorption with thymocyte antigens eliminates this cytotoxic response completely.

No cytotoxic response was detected using "non-immune"normal rabbit serum.

Table 1. Indirect fluorescence of rat kidney sections following incubation with anti-brushborder or anti-thymocyte antibodies.

previous absorption of the antibodies with:	*anti-brushborder antibody*	*anti-thymocyte antibody*	*normal rabbit serum*
standard[b])	+	+	-
Bb-ag[c])	-	-	-
Thym-ag[d])	+	-	-

Cryostat kidney sections were incubated with either anti-brushborder or anti-thymocyte antibodies and FITC conjugated goat anti-rat IgG, following absorption with various tissue antigens.

a) +: positive staining; -: no detectable staining.

b) all antibodies used were subsequently absorbed to rat heart tissue extracts and rat erythrocytes which procedure is referred to as standard.

c) Bb-ag:: brushborder antigens.

d) Thym-ag: thymocyte membrane antigens.

MIF assays.

Normal rat thymocytes are inhibited in their migration in vitro upon contact with heterologous anti-brushborder globulin reflected in a dose-related migration inhibition, whereas control heterologous antisera such as rabbit anti-rat albumin or normal rat IgG were inactive in this respect (not shown here) [17]. As can be seen from Figure 2A, this response can be reduced after absorption with both brushborder antigen as well as thymocyte membrane antigens, although absorption with brushborder antigen appeared to reduce the activity

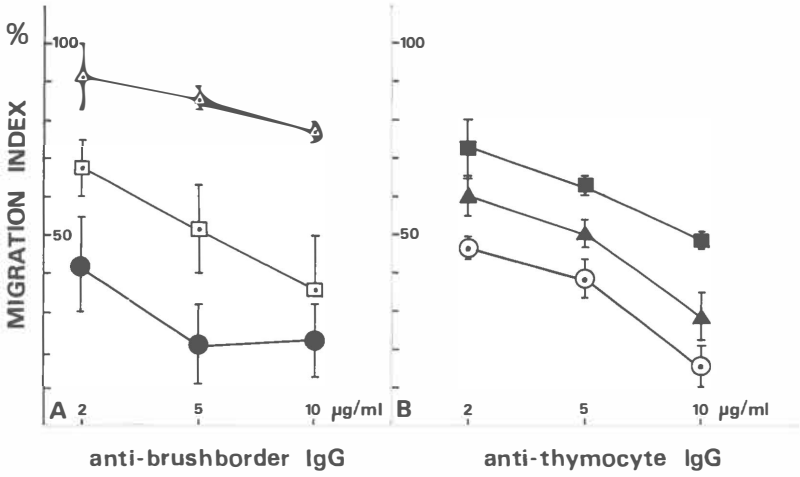


Fig. 2. Direct migration inhibition assay of normal rat thymocytes after stimulation with anti-brushborder or anti-thymocyte antibodies. Dose-related migration inhibition response upon stimulation with A) anti-brushborder IgG (●-●); anti-brushborder IgG after absorption with thymocyte antigens (□-□); anti-brushborder IgG after absorption with brushborder antigens (▲-▲). B) anti-thymocyte IgG (0-0); anti-thymocyte IgG after absorption with thymocyte antigens (■-■); anti-thymocyte IgG after absorption with brushborder antigens (▲-▲). Symbols represent arithmetic means of migration indexes ± standard deviations of three pools of rat thymocytes from 5 rats per pool.

a great deal, whereas absorption with thymocyte membrane antigens only partly reduced this inhibitory response upon this antibody. Anti-thymocyte globulin induces also a dose-related migration inhibition;in this case the thymocyte membrane antigen preparation appeared to be the most efficient immunoabsorbent to reduce the MIF response, whereas brushborder antigen did remove the activity to a lesser extent from this test material (Fig. 2B). For comparison with the cytotoxicity results a summary of the migration inhibition data is included in Table 2 (symbols in parentheses).

Table 2. Lysis of normal rat thymocytes in vitro following incubation with either anti-brushborder or anti-thymocyte antibodies and complement.

previous absorption of the antibodies with:	*anti-brushborder antibody* [a]	*anti-thymocyte antibody*	*normal rabbit serum*
standard [b]	+ (+)	++ (+)	-
Bb-ag [c]	- (-)	± (±)	-
Thym-ag [d]	- (±)	- (-)	-

a) + indicates clear cytotoxicity response (50 % lysis at 1/16 dilution); ++ 50 % lysis at 1/128 dilution; ± reduced cytotoxicity response (≤ 50 % lysis at 1/2 dilution); - no detectable cytotoxicity response (≤ 10 % lysis at 1/1 dilution).
b), c) and d) as indicated under table 1.
Symbols in parentheses are given for comparison with the migration inhibition data of Fig. 2: + = migration inhibition; ± = moderate reduced migration inhibition; - = strongly reduced migration inhibition.

DISCUSSION

The data presented in this paper suggest the coexistence of two specificities present in both heterologous anti-brushborder antibodies as well as heterologous anti-thymocyte antibodies; i.e., anti-brushborder serum contains also anti-thymocytes specificity, whereas anti-thymocyte serum contains also anti-brushborder specificity. Evidence for this finding was obtained by using immunofluorescence techniques on cryostat kidney sections (Fig. 1 and Table 1), cytotoxicity assays in vitro (Table 2) and migration inhibition assays using rat thymocytes (Fig. 2).

The question arises whether only cross-reacting antibodies or two distinct specificities are involved in the antibodies studied. Although the immunofluorescent as well as the cytotoxicity results show some unexplained features, these data as well the migration inhibition results (Fig. 2) suggest that two specificities rather than only cross-reacting antibodies are involved. Thus a dose-related migration inhibition induced in both of the antisera upon thymocytes can best be reduced by absorption of the antisera with their own antigens while absorption of anti-brushborder serum with thymocyte antigens or absorption of anti-thymocyte serum with brushborder antigens results in a partial reduction of the activity of the respective antisera.

These selective absorption data are in conflict with the assumption that only one cross-reacting specificity is present in both of the antisera tested. However, it cannot be concluded from these data whether the overlap of the cross-absorption results is due to the limited resolution ofthe test system or to cross-reacting specificities present in the antisera tested (or to both of these possibilities).

In contrast to the MIF data, the cytotoxicity results show some discrepancy with respect to absorption studies (Table 2). The fact that absorption with both brushborder antigens as well as with thymocyte antigens results in a complete disappearance of the cytotoxic activity of anti-brushborder serum remains obscure. Since the absorption procedure used resulted in highly reproducible data, irregularities due to the absorption steps should be excluded as an explanation for this discrepancy. It might be speculated that only anti-thymocyte specificities mediate the cytotoxic activity of both of the antibodies studied in contrast to the migration inhibition response which is mediated by both the anti-thymocyte as well as the anti-brushborder specificities.

Whatever the explanation might be, the cause of the dual specificity present in the antisera studied remains to be elucidated. If immunologic cross-reactivity of antibodies raised to tissue antigens does play a major role, this dual specificity may be caused by shared antigens present in the kidney and on the thymocyte membrane. Indirect evidence supports this idea. For instance while brushborder antigens elicited anti-thymocyte specificity in rabbits, several other tissue

extracts including liver homogenate and choroid plexus homogenate did not (unpublished results). BALCH et al. [20] described the disappearance of heterologous anti-T-cell antibody activity following absorption with rat kidney homogenate indicating also that some kidney tissue structure might specifically bind anti-T-cell specificities in contrast to other tissue homogenates. Moreover we did not observe any "classical" anti-GBM antibodies in our anti-thymocyte serum, which might be expected when impurities would play a major role in the generation of cross-reacting antibodies as is described in heterologous anti-thymocyte serum raised to human thymocytes [167, 158, 156]. Therefore in this serum a thymocyte membrane antigen may have given rise to the anti-brushborder antibodies observed, rather than connective tissue contaminants which are usually claimed to elicit anti-GBM antibodies.

In view of the fixed antigen concept of glomerular immune complex formation [57, 92, 54, 130] a possible sharing of glomerular antigenic structures with T-cell determinants may be involved in the pathogenesis of immune complex glomerulopathies. Studies as to the question whether our heterologous anti-thymocyte specificity plays a role in the pathogenesis of the heterologous immune complex glomerulopathy in the rat are in progress.

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES.

III. Binding of anti-Fx1A antibodies in glomeruli is dependent on dual specificity.

Wilma M. BAGCHUS, J.T.W.M. VOS, PH.J. HOEDEMAEKER , W.W. BAKKER.

Department of Pathology, University of Groningen,
Groningen, The Netherlands.
Department of Pathology, University of Leiden,
Leiden, The Netherlands.

SUMMARY

Rabbit antibodies against rat tubular brushborder antigens (Fx1A) give rise to in situ formation of immune aggregates along the glomerular capillary walls after intravenous injection into rats. These antibodies (anti-Fx1A), able to produce heterologous immune complex glomerulopathy (HIC) in the rat, have previously been shown to bind with brushborders (anti-BB) as well as with rat thymocytes (anti-T). In the present communication, this dual specificity was also demonstrated in antibodies eluted from kidneys of rats with HIC. It further appeared that, when the anti-thymocyte binding activity was selectively removed from these antibodies, using immunoabsorption with rat tissue extracts, these anti-Fx1A antibodies were no longer able to stain glomerular basement membranes (GBM) as demonstrated at the ultrastructural level using the peroxidase technique. Following perfusion of these antibodies in the normal rat kidney ex vivo, binding along the capillary walls was also below the detection level, in contrast to non anti-T depleted anti-Fx1A IgG. Biochemical analysis (including immunoblotting) showed that the anti-T moiety of anti-Fx1A

was directed to a 90 kD component of FxlA, since selective absorption of this specificity prevented staining of this 90 kD component.
It is concluded that this anti-T specificity within rabbit anti-FxlA plays a crucial role in local immune complex formation in the rat kidney ex vivo. Whether this holds also for its role in the pathogenesis of HIC in vivo awaits further confirmation.

INTRODUCTION

Administration of rabbit antibodies against rat tubular brushborder antigens (FxlA) causes immediate formation of immune aggregates along the glomerular capillary walls [21, 57, 67]. The resulting heterologous immune complex glomerulopathy (HIC) (as well as the autologous Heymann nephritis) have been considered as an experimental counterpart of human membranous glomerulopathy (57, 67, 70, 89, 146). The nephritogenic heterologous antibody (anti-FxlA) has been shown to contain anti-brushborder (anti-BB) as well as anti-T cell specificity (anti-T) [18, 19].
Since also rabbit anti-thymocyte IgG is able to bind along the glomerular basement membrane (GBM) [9] , the question arises whether anti-T activity associated with anti-FxlA IgG plays a role in immune complex formation in HIC. Therefore, we now report on the binding of rabbit anti-FxlA IgG (including anti-FxlA eluted from kidneys of rats with HIC), with thymocytes or kidneys from normal rats using immunofluorescence (IF). Anti-FxlA was absorbed with rat tissue extracts in order to remove anti-T cell activity and the binding of this anti-T depleted antibody was studied in vitro upon normal rat kidney sections as well as after ex vivo perfusion into the normal rat kidney.
In addition biochemical analysis (immuno-blotting) was carried out to study the component(s) of FxlA to which the anti-T cell specificity of anti-FxlA IgG might be directed.

MATERIALS AND METHODS.

Animals. Female Wistar rats of three months of age were used throughout the study.The rats were fed with RMBH (Woerden, The Netherlands) and received water ad libitum.

Preparation of FxlA and rat tissue extracts. FxlA antigens were prepared from the cortices of normal Wistar rat kidneys, as described by Edgington et al [65]. Rat-liver, -heart, -lung, -spleen and -thymus extracts and bovine-heart tissue-extract were prepared in phosphate buffered saline (PBS) of 4C using a warring blendor. The extracts were dialysed against distilled water for 72 h, lyophilized and stored at 4C [19].

Preparation of rabbit antibodies. Rabbit antisera against FxlA were prepared and tested as described previously [67]. IgG was purified by ion-exchange chromatography on diethylaminoethyl sephadex (DE 52,Whatman, Kent, England) with 0.01M buffered saline (pH 8.0) as elution buffer. Subsequently, this IgG was absorbed to sonicated rat glomeruli as described by Feenstra et al. [67], dialysed against PBS for 72 h, adjusted to a final concentration of 10 mg/ml and stored at - 20C until use. Rabbit anti-rat albumin IgG (R-a Alb) was prepared and tested for specificity as described earlier [9].

Absorption of heterologous anti-FxlA IgG with tissue antigens. Rabbit anti-FxlA IgG was routinely absorbed with packed rat erythrocytes (1 ml of 100μg IgG with 0.3 ml packed erythrocytes for 60 min at 4C) and subsequently with washed liver tissue extract (20 mg extract per ml IgG solution containing 100μg IgG for 3 h at room temperature followed by absorption overnight at 4C) to remove non-specific "anti-rat" antibodies.
Antibodies treated by this routine absorption procedure are referred to as anti-FxlA IgG. One batch of anti-FxlA IgG was depleted from thymocyte binding antibodies by absorption with rat heart tissue (RHT) (2 ml of 300 mg RHT mixed with 1 ml of 100μg IgG) for 3 h at room temperature and 18 h at 4C. This antibody is referred to as RHT-absorbed anti-FxlA IgG. RHT extract was used, since comparative pilot studies with various rat tissue extracts, including RHT, indicated

that the latter material was able to absorb anti-thymocyte binding activity without affecting the anti-BB titre significantly: heart tissue extract from other species (ox, sheep) appeared negative in this respect. To exclude alterations in binding activity due to the absorption procedure, anti-Fx1A IgG was absorbed using equal amounts of bovine heart tissue (BHT) extract prepared in an identical manner as described for RHT. This antibody is referred to as BHT-absorbed anti-Fx1A IgG. The absorbed antibodies were repurified by ion-exchange chromatography.

After absorption, the presence of anti-T activity and anti-BB titres in the IgG fraction were tested using IF as described below. To study the specificity of antibodies removed by RHT absorption, 25 g RHT extract, used for absorption of heterologous anti-Fx1A IgG, was washed five times with PBS. The pellet was incubated with 125 ml 0.05 M citrate buffer and stirred for 4 h at room temperature; after centrifugation (15 min, 3,000g) the supernatant was adjusted to pH 7.2 with 0.1M NaOH, dialysed against Tris-buffer for 72 h and run over a DE 52 column. The IgG fraction of these heart eluted antibodies were tested for the presence of anti-T activity and anti-BB activity using IF.

Immunoelectron microscopy (IEM) of normal rat kidney after incubation with anti-Fx1A. Cryostat sections (20 μ) of normal rat kidney were washed four times with PBS, pH 7.2, and incubated with 0.5 ml rabbit anti-Fx1A IgG. Either anti-Fx1A IgG, RHT-absorbed or BHT-absorbed anti-Fx1A IgG of equal anti-brushborder titre were used for these incubations. Peroxidase method, described by Hoedemaeker et al. [91], was carried out with minor modifications. Briefly: after washing with PBS, the kidney sections were incubated with 1 ml goat anti-rabbit (GAR) IgG (50 mg/ml), washed five times, incubated with 1 ml rabbit anti-peroxidase (1.25 mg/ml), washed five times with PBS. Subsequently, 1 ml horseradish peroxidase (Boehringer, 37 μg/ml) was added, followed by washing with PBS five times. Finally, 1 ml 3.3 diaminobenzidine tetrahydrochloride (2 mg/ml), containing 0.01% H_2O_2, was added and the reaction was stopped by post-fixation in 1 % phosphate buffered OsO_4. All incubation steps were performed at room temperature for 30 minutes. After postfixation for 60 minutes at 4C, sections were processed according to standard methods [91].

Ex vivo perfusion with anti-FxlA IgG. Unilateral perfusion of kidneys was performed as described by van Damme et al. [57] with minor modifications as described elsewhere [9]. After mobilization of the left kidney and appropriate ligations, the renal vein was punctured and the kidney was perfused with PBS, pH 7.2, of 37 C (2 ml/min). Absorbed anti-FxlA IgG (10 ml; anti-BB titre 1/128) was perfused, followed by perfusion with 5 ml PBS of 37 C to remove unbound antibodies or immune complexes or both. Either anti-FxlA IgG, or RHT-absorbed or BHT -absorbed anti-FxlA IgG solutions were used in the perfusion experiments . Specimens of the perfused kidneys were prepared for light and fluorescence microscopy.

Elution of anti-FxlA from kidneys of rats with HIC. HIC was induced in 6 Wistar rats by intravenous injection with 5 mg rabbit anti-FxlA IgG [57]. After 3 days the rats were anaesthetized and their kidneys were perfused with 0.9 % NaCl, to remove circulating antibodies, and snap frozen in freon 12 at - 80C. Elution of antibody from these kidneys was performed according to Woodroffe & Wilson [178]. Briefly, for each elution experiment 5 μ cryostat section (from two kidneys) were collected and washed five times with PBS, pH 7.2, by centrifugation (10 min, 150 g) to remove non-fixed antibody. The elution was done with 15 ml 0.05 M citrate buffer (pH 3.2) and stirred for 3 h at room temperature. After centrifugation (15 min, 300 g) the supernatant was neutralized with 0.1 M NaOH and dialyzed against PBS for 48 hours and assayed for protein concentration using spectrophotometry at 280 nM. Eluates were concentrated (Amicon B15) to 10 mg/ml before analysis.

Analysis of anti-brushborder and anti-thymocyte activity by IF. Anti-brushborder or anti-thymocyte specificity of anti-FxlA IgG was assayed using standard IF methods upon either normal cryostat sections of rat kidney or freshly isolated rat thymocytes, respectively. FITC-conjugated GAR IgG (Nordic, Tilburg, The Netherlands) was used as a second step. Incubation of tissue or cells with rabbit anti-albumin IgG and FITC-conjugated GAR was carried out as a control. Indirect membrane IF upon thymocytes was done according to standard techniques. Briefly, 2 x 10^6 thymus cells were incubated with 0.05 ml heterologous antibodies or eluate for 30 min at 4C, washed and subsequently incubated with 0.05 ml FITC-GAR, washed again, resuspended in mounting

medium containing 10^{-3} M p-phenylene-diamine. Cells were examined under a Leitz Orthoplan fluorescence microscope with Ploemopak illumination, equipped with phase contrast filters allowing direct comparison of IF and light microscopy patterns.

Immunoblotting. Samples of FxlA (50 mg/ml) were separated by electrophoresis on polyacrylamide gels (4-30%) containing SDS (SDS-PAGE), using Laemmli's buffer system [110]. Immuno-blotting was carried out according to Towbin et al [170] with minor modifications. After transfer of separated proteins to nitrocellulose sheets, they were saturated with bovine serum albumin (BSA) 1% followed by incubation with 100 μg/ml of either anti-FxlA IgG or RHT-absorbed anti-FxlA for 2 h. Subsequently they were washed with PBS pH 7.3 (3 times) and the sheets were incubated with peroxidase conjugated swine-anti-rabbit IgG (1/500, Dakopatts, Copenhagen, Denmark) for 1 hour. Peroxidase was detected by incubation with amino-ethyl carbazol (Aldrich, Milwaukee, USA), containing 0.01% H2O2. The reaction was stopped by washing with distilled water. Marker proteins were detected by staining with amido-black for 5 minutes.

RESULTS

Either rabbit anti-FxlA IgG or BHT-absorbed anti-FxlA IgG was able to stain both tubular brushborders and GBM of the normal rat kidney as well as fresh rat thymocytes using IF (table 1). Following absorption with RHT, the ability to stain thymocytes was abolished completely, whereas the staining of brushborders was clearly detectable (table 1). Results from the elution experiments showed that antibodies eluted from kidneys of rats with HIC stained brushborders and glomeruli as well as thymocytes after incubation with rat kidney sections or thymocytes, respectively (Figs 1 & 2). Also from RHT which had been used for immunoabsorption of anti-FxlA, both brushborder as well as thymocyte staining antibodies could be eluted. Reaction product was detected along brushborders of proximal tubules after incubation of kidney tissue with either BHT-absorbed or RHT-absorbed anti-FxlA IgG

TABLE 1

Immunofluorescence of rat kidney or thymocytes after incubation with heterologous anti-Fx1A IgG and FITC-labeled goat-anti-rabbit IgG.

	Membrane staining of fresh rat thymocytes 2)	*Staining of tubular brushborder in rat kidney sections* 2)
Rabbit anti-Fx1A IgG (100 µg/ml) 1)	+	+
Rabbit anti-Fx1A IgG 100 µg/ml) RHT-absorbed)	-	+
Rabbit anti-Fx1A IgG 100 µg/ml) (BHT-absorbed)	+	+

1) Rabbit anti-Fx1A was incubated at room temperature with either normal rat cryostat sections or at 4 C with rat thymocytes as described in section material and methods. GAR-FITC was used as second incubation step. All anti-Fx1A IgG was absorbed with rat erythrocytes and liver tissue extract; absorption to rat heart tissue (RHT) or bovine heart tissue (BHT) was done as described in the materials and methods section.
2) Clear IF staining of either thymocyte membranes or tubular brushborders is designated + ; no detectable fluorescence is designated with -.

of identical anti-BB titre (Fig. 3A,B). Reaction product could also be detected throughout the GBM and adjacent epithelial cell membranes after incubation with BHT-absorbed anti-Fx1A (Fig 3c). In contrast, following incubation with RHT-absorbed antibody, no staining of these sites could be seen at all (fig 3d).

Localization of rabbit IgG along the GBM could be clearly seen after perfusion of BHT-absorbed anti-Fx1A IgG (anti-BB titre 1/128) into the normal rat kidney ex vivo (fig 4).

Perfusion of equal amounts of anti-Fx1A IgG with identical anti-BB titre resulted in a similar localization.

In contrast, equal amounts of RHT-absorbed anti-Fx1A IgG of identical titre (1/128) did not show any detectable IF staining of rabbit IgG in the glomeruli of perfused rat kidneys.

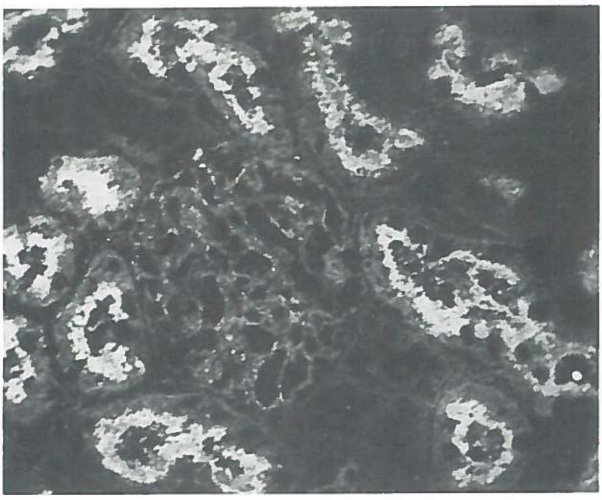


Fig 1. Indirect immunofluorescence of a normal rat kidney tissue section after incubation with antibodies eluted from a kidney of a rat with HIC, followed by incubation with FITC-conjugated GAR. Fluorescence of the brushborder of the proximal tubules is prominent. In addition, staining of the capillary wall of the rat glomerulus is observed. x 350.

The results of the immuno-blotting are shown in figure 5. It can be seen that while no background staining is present after incubation with only swine-anti rabbit IgG (lane A), anti-Fx1A stains several components of the Fx1A molecule, including two major bands reflecting components of approximately 330 kD and 90 kD respectively (lane B). In contrast, following incubation with anti-T depleted anti-Fx1A an altered staining pattern can be seen: the 90 kD band as well as several minor bands can no longer be detected (lane C). Lane M shows the marker proteins.

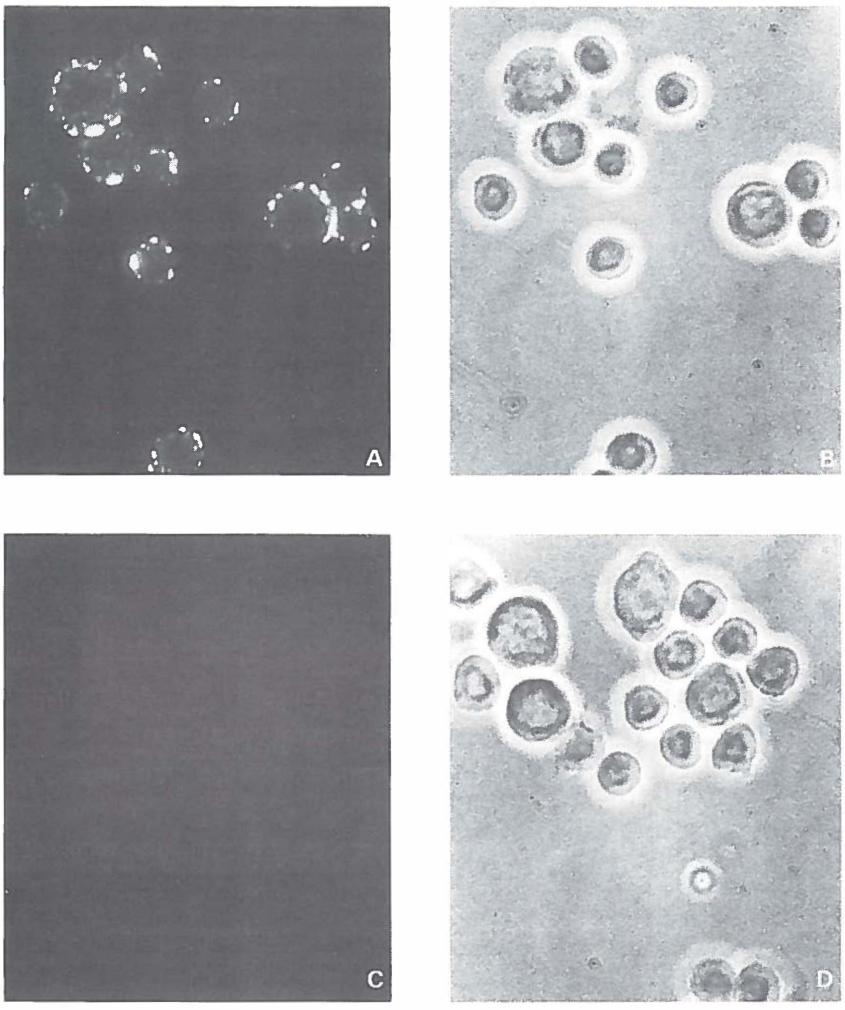


Fig 2. Typical patchy membrane fluorescence of rat thymocytes following incubation with antibodies eluted from kidneys of a rat with HIC and GAR-FITC (A). From the corresponding phase-contrast micrograph (B) it can be seen that all thymocytes present are staining; in cell suspensions incubated with control R-a Alb IgG and GAR-FITC no staining can be observed (C&D). x 1,200.

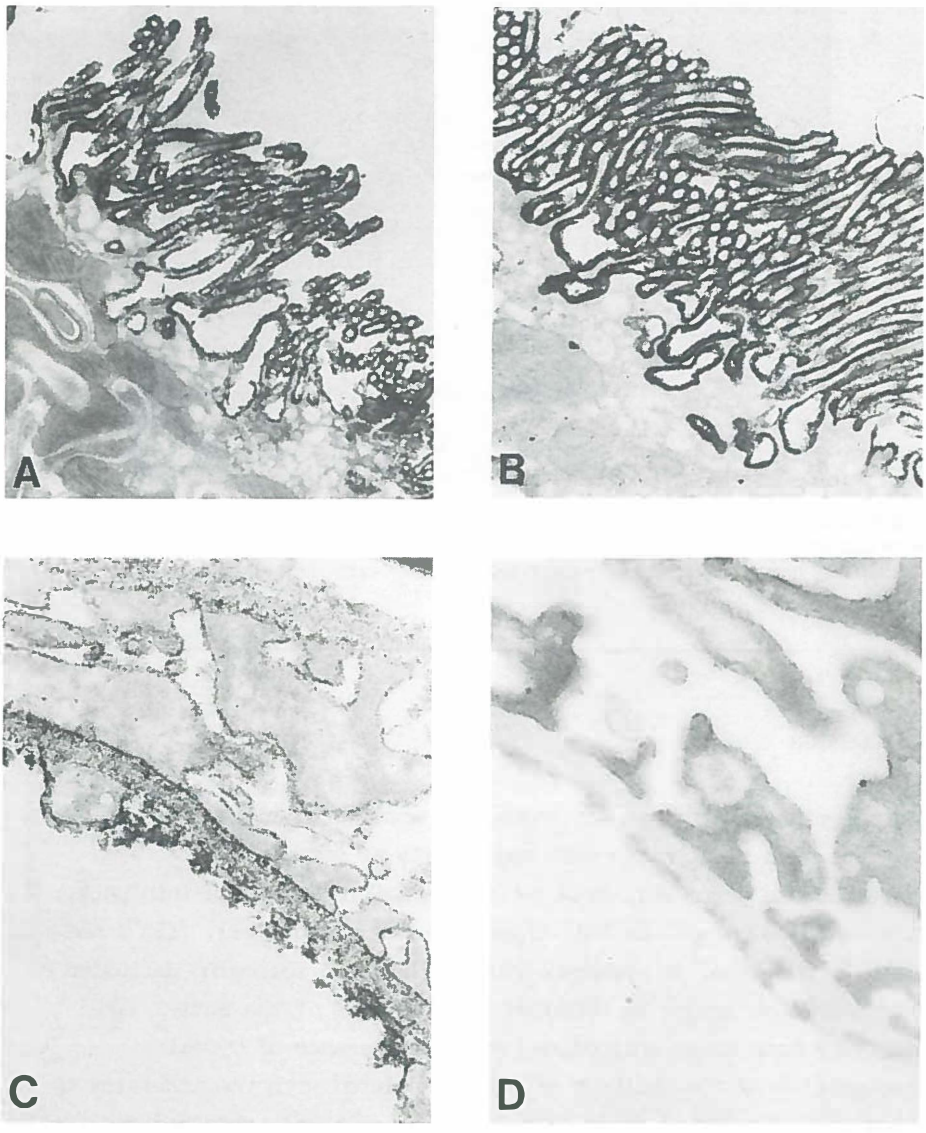


Fig 3. Immunoelectronmicrograph of normal rat kidney after incubation with BHT absorbed anti-Fx1A IgG (anti-BB titre 1/128, A&C) or RHT absorbed anti-Fx1A IgG (anti-BB titre 1/128, B&D). After incubation with BHT absorbed anti-Fx1A IgG reaction product is present along the brushborder of proximal tubules (A), and throughout the GBM and in parts of epithelial and endothelial cell membranes adjacent to the GBM (C). Similar staining of brushborders can be seen using RHT absorbed anti-Fx1A (B), whereas staining of GBM or membranes of GBM lining cells is no longer detectable. A&B x 23,664; C & D x 27,250.

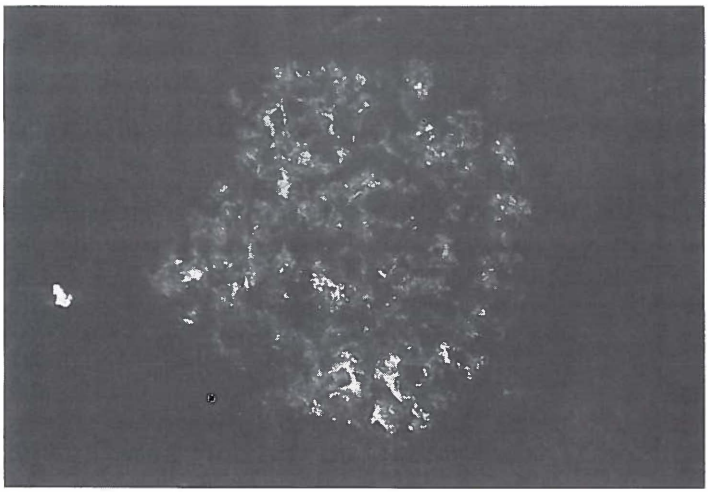


Fig 4 Direct immunofluorescence of a normal rat kidney perfused with BHT-absorbed anti-Fx1A IgG (125 µg/ml, anti-BB titre 1/128, flow 2 ml/min) stained with FITC-conjugated GAR. Fine granular deposits of rabbit IgG along the capillary wall can be observed. x 440.

DISCUSSION

In the present study it was shown that antibodies eluted from kidneys of rats with HIC contain dual specificity as described for heterologous serum anti-Fx1A antibodies before injection into rats, i.e. anti-BB as well as anti-thymocyte specificity [18], (fig 1 and 2a). In addition, it appeared that localization following perfusion ex vivo could no longer be detected after removal of the anti-T like activity from these antibodies. The disappearance of T-cell recognition of the antibody is also associated with the inability to bind with the GBM in vitro (fig 3). Thus, routinely absorbed anti-Fx1A IgG shows a similar binding as described by van Damme et al. [57] for heterologous anti-Fx1A using IEM (fig 3c) which disappears after absorption with thymocyte extracts or RHT (fig 3d).
As stated in 'Materials and Methods' anti-T activity within anti-Fx1A was removed using absorption with RHT rather than with thymocytes. Although thymocyte membrane extract used as immunoabsorbents gave

identical results, RHT was preferred for practical reasons related to the relatively large amount of tissue extract needed for each absorption procedure (As will be pointed out below, the removed anti-T specificity probably recognizes an antigen present in heart tissue as well as in thymus cell membranes).

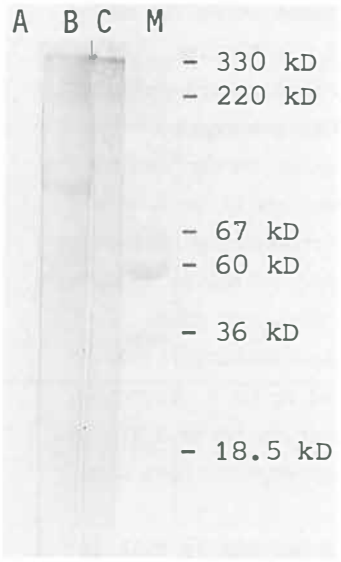


Fig 5 Immunoblotting experiment: 50 µg of Fx1A was separated by SDS-PAGE and transferred to nitrocellulose sheets, which were incubated with either PBS (lane A), anti-Fx1A IgG (lane B) or anti-T depleted anti-Fx1A (Lane C), followed by peroxidase labelled swine anti-rabbit IgG (SaRaIgG). The bands represent bound SaRaIgG, detected with aminoethylcarbazol. No background staining is detected in lane A. Lane B shows two major bands, one at 330 kD and the other at 90 kD. In lane C, the 90 kD band can no longer be detected, but the 330 kD band is still present. Lane M shows protein staining (amido-black) of the marker proteins. Marker proteins used were ferritin (18.5 kD), lactate dehydrogenase (36 kD), catalase (60 kD), albumin (67 kD), ferritin (220 kD) and thyroglobulin (330 kD).

The question can be raised, whether the eluted anti-Fx1A is originating from the glomeruli exclusively, or also from other parts of the kidney, i.e. tubular brushborders. Sections from saline perfused kidneys of rats with HIC, used for elution, do not show rabbit IgG outside the glomeruli, suggesting that the eluted antibodies originate from glomeruli rather than from other parts of the kidney, although some contamination cannot be excluded completely. Since renal tubular antigen Fx1A is impure material containing many epitopes, polyclonal anti-Fx1A consequently may contain many specificities, including anti-T like specificity. Although this remains to be confirmed in vivo, it appears from the present data that the nephritogenicity of anti-Fx1A is dependent on the presence of anti-T like paratopes.

Other authors have also observed that anti-brushborder antibodies per se are not necessarily nephritogenic (Kerjaschki et al. [110], Tauc et al. [1985], Miettinen et al, [119]). We asked ourselves the question to which epitope the anti-T like moiety in our anti-FxlA IgG is directed. Therefore, immunoblotting experiments were carried out. The data confirm the importance of two major constituents of FxlA antigen, i.e. GP 90 and GP 330 ,as described by Ronco et al.[139] and Kerjaschki & Farquhar [105]; the latter constituent being the major antigen involved in autologous Heymann nephritis in the rat [105]. While these two glycoproteins are stained with rabbit-anti-FxlA IgG, anti-T depleted anti-FxlA stained only the 330 kD component, indicating that GP 90 is associated with an epitope recognized by the anti-T specificity within anti-FxlA. This assumption is in line with data of Ronco et al. [139], who observed nephritogenicity of (mouse) monoclonal anti-rat brushborder antibodies (MoAb) following injection into rats. This MoAb , which stained rat heart tissue , also recognized a 90 kD molecule of FxlA using immuno-blotting [139]. In addition in our hands this MoAb (kindly provided by Dr.P. Verroust, Paris) stained also rat T-cell areas ,in contrast to three different MoAb against GP 330 which were negative in this respect (also kindly provided by Dr.P.Verroust), [141].
From these results it is likely that the T-like epitope in FxlA is very similar if not identical with the GP 90 molecule recognized by heterologous anti-FxlA, although further investigation has to be carried out concerning the role of other FxlA components, reflected in the disappearance of some minor bands (between 18.5 and 60 kD) concomitantly with GP 90, following incubation of anti-T depleted anti-FxlA (fig 5).
It is clear that removal of this anti-T specificity reduces the binding properties of anti-FxlA to glomerular structures in vitro and after perfusion ex vivo (fig 3d). The reason for this is unclear. The possibility that altered binding capacity of anti-FxlA following depletion of anti-T (or anti-GP 90) specificity is due to changes in electrical charge of the molecule can be excluded since all IgG was purified by ion exchange chromatography in an identical way.
It is concluded that anti-T like paratopes in heterologous anti-FxlA IgG may play a crucial role in glomerular immune complex formation in

situ and possibly also in the pathogenesis of HIC although this awaits confirmation in vivo.

Studies are in progress showing glomerular damage and massive proteinuria following administration of (mouse) anti-T MoAbs suggesting also a role for certain anti-T paratopes in experimental glomerulopathy in the rat.

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES.
V. Immunohistochemical characterization of the anti-T cell specificity of heterologous anti-Fx1A antibodies.

WM Bagchus[1], JTWM Vos[1], PhJ Hoedemaeker[2], EHG van Leer[3] & WW Bakker[1]

1) Department of Pathology, University of Groningen, Groningen
2) Department of Pathology, University of Leiden, Leiden, The Netherlands
3) Unite Inserm U 64, Hopital Tenon, France.

SUMMARY

Administration of rabbit antibodies (Abs) directed against rat brushborder antigens (Fx1A) results in heterologous immune complex glomerulopathy (HICN), caused by an immediate in situ formation of subepithelial immune complexes (ICxs). In previous studies two major specificities were observed in these anti-Fx1A Abs: anti-GP 330 and anti-GP 90. In contrast to autologous immune complex glomerulopathy, in which anti-GP 330 Abs has been shown to play a major role in glomerular ICx formation, in HICN anti-GP 90 Abs may also be important in subepithelial ICx formation. To elucidate the specificities of rabbit-anti-Fx1A we compared the staining patterns of these Abs with monoclonal Abs (Moabs) directed against GP 330 and GP 90 by immunohistochemical staining on lymphoid organs of the rat. The (almost) identical staining patterns of anti-Fx1A IgG and anti-GP 90 Moab upon spleen, lymph node and thymus sections, and the absence of lymphoid staining using anti-GP 330 Moabs confirm previous data showing anti-GP 90 specificity in rabbit anti-Fx1A by immunoblotting techniques.

INTRODUCTION

Intravenous administration of heterologous antibodies directed against rat brushborder antigens (Fx1A) results in heterologous immune complex glomerulonephritis (HICN). The pathogenetic mechanism of this experimental glomerulonephritis has been shown to be an immediate in situ formation of subepithelial immune complexes [57, 54].
Since Fx1A is a mixture of various brushborder antigens, polyclonal anti-Fx1A antibodies are directed to a heterogenous set of epitopes. In previous studies we demonstrated at least two major specificities. First anti-GP 330 activity which stained the brushborder and is directed against an antigen thought to be the antigen involved in Heymann nephritis [105, 106], and secondly an anti-GP 90 antibody directed to an antigen present on e.g. brushborder and thymocytes [19]. In earlier studies we were able to show that this GP 90 antigen is also involved in the pathogenesis of HICN [14]. This was confirmed by studies of Ronco et al. [139].
To further elucidate the specificity of heterologous polyclonal anti-Fx1A, we compared immuno-histochemical staining patterns of this antibody on lymphoid organs of the rat with that of monoclonal antibodies directed against GP 330 and GP 90 respectively.

MATERIAL AND METHODS

Antibodies. Conventional rabbit antibodies against Fx1A , containing both anti-GP 330 and anti-GP 90 specificity, were prepared according to the method described by Feenstra et al. [67]. Pilot studies using various rat tissue extracts as immunoadsorbents have shown that rat heart tissue homogenate [RHT] (besides rat thymus homogenate) is able to remove efficiently the anti-T specificity. Therefore, in this study anti-T-activity present in anti-Fx1A IgG was absorbed using RHT as described earlier [14]. This IgG fraction is referred to as anti-Fx1A (-T).
The anti-T antibodies absorbed to RHT were recovered by elution with 0.5 M citrate buffer pH 3.2 (15 ml buffer mixed with 5 mg absorbent) for 5 hours as described earlier [14]. This fraction is referred to as heart eluate IgG. For immuno-histology staining the absorbed and

nonabsorbed IgG fractions of polyclonal antibodies were adjusted to a concentration with anti-brushborder activity with an end point dilution of 1/64, shown by IF, (50 μg/ml IgG for anti-Fx1A IgG, 200 μ g/ml for anti-Fx1A (-T) IgG and heart eluate IgG).
Monoclonal antibodies against GP 90 and GP 330 epitopes of Fx1A were a gift from Prof. Dr.P. Verroust, Paris. Immunoprecipitation indicated that MoAb F2/8 is specific for GP 90, MoAb F1/12, F3/28 and F3/237 are specific for three different epitopes of GP 330. Details of the production, characterization and specificity analysis of these Moabs have been reported previously [139],[140],[43]. Affinity purified rabbit-anti-RTE α5 IgG (a gift from Dr. E.de Heer, Leiden) and rabbit anti GP 330 serum (a gift from Dr.D. Kerjaschki, Austria) were used in a concentration of respectively 50 μg/ml and 1:5 serum dilution. Since limited amounts were available, these antibodies were only used for staining of spleen sections.

Tissue preparation. Lymph nodes, in toto embedded in Tissue Tek-II (Miles, USA), and tissue blocks of spleen and thymus of a normal Wistar rat were snap frozen in freon 12 of -100 C and stored at -80C until use. Cryostat sections were cut at 4 μ and air-dried for 20 minutes with a hairdrier. Subsequently, slides were fixed in acetone for 10 minutes at room temperature and washed in phosphate buffered saline (PBS) pH 7.4.

Immuno-histology staining. A two step indirect immunoperoxidase technique was used, according to standard procedures [169]. Tissue sections were incubated with 25 μl polyclonal antibody (50 or 200 μg IgG/ml, depending on the anti-brushborder titre) or monoclonal antibody (10 μg IgG/ml) for 30 minutes, washed and subsequently incubated with 25 μl of a 1:40 dilution of horseradish peroxidase conjugated swine anti-rabbit IgG or a 1:20 dilution of horse radish peroxidase conjugated goat anti mouse IgG (Dakopatts, Copenhagen, Denmark) supplemented with 1% normal rat serum, for 15 minutes. After washing in PBS, 3-amino-9-ethyl-carbazole (Sigma, St.Louis, Missouri, USA), together with H_2O_2, was used as a substrate for the demonstration of peroxidase reactivity according to the method of Graham,Lundholm & Karnovsky [78]. After washing in distilled water, counterstaining of the sections was performed by incubation in fresh

haemalum for about 30 seconds. The sections were mounted with glycerol-gelatin (Merck, Darmstadt, FRG).

RESULTS

Spleen

The results of the staining patterns on normal spleen tissue after incubation with various anti-Fx1A antibodies are summarized in table 1, and illustrated in fig 1. Heterologous anti-Fx1A IgG (Fig 1A) and rabbit anti-RTE α5 stains T-cell areas and reticuloendothelial lining cells of sinusoids in the red pulp. The same staining pattern was observed using heart eluate IgG. In contrast, anti-Fx1A (-T) did not stain these structures (fig 1B).

Table 1
Staining patterns on spleen tissue after incubation with polyclonal or monoclonal anti-Fx1A antibodies

	T-cell areas	*Sinusoids*	*B-cell areas*
anti-Fx1A	+	+	-
anti-Fx1A (-T)	-	-	-
Heart eluate	+	+	-
Anti-RTE α5	+	+	-
Anti-GP 330	-	-	-
Moab F2/8 (a-GP 90)	+	+	-
Moab F1/12 (a-GP 330)	-	-	-
Moab F3/28 "	+/-	-	-
Moab F3/237 "	-	-	+

+ = positive staining, - = no staining +/- staining of a few cells.

Monoclonal anti-GP 90 (fig 1C) shows an identical staining pattern as observed after incubation with polyclonal anti-Fx1A IgG (fig 1A). Polyclonal and monoclonal antibodies (fig 1D) directed against GP 330, do not show staining of T-cell areas or sinusoid lining cells, except for Moab F3/28 which stained a few cells in the T-cell area (table 1), whereas Moab F3/237 stained B-cell areas. In all spleen sections endogenous peroxidase activity of macrophages is observed. This is best observed in fig 1B and 1D where no other spleen structures are stained.

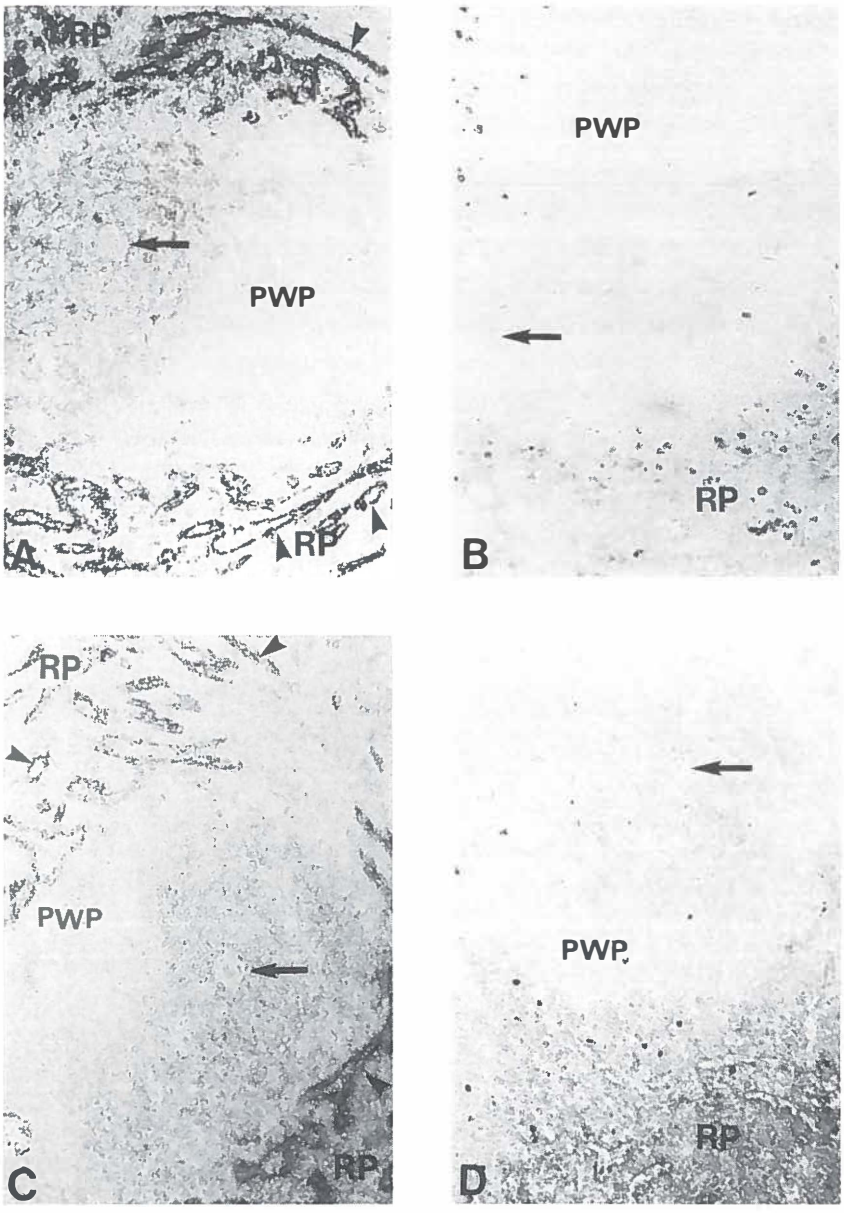

RP
PWP
RP
A
PWP
RP
B
RP
PWP
RP
C
PWP
RP
D

Fig 1A: Spleen section stained with anti-Fx1A IgG (50 µg/ml). T-cells in peri-arteriolar lymphocyte sheath (PALS) (arrow) are stained. No staining of B-cells in peripheral white pulpa (pwp) is observed. Strong staining of sinusoidal lining cells of red pulpa sinusoids (arrowheads) is present (x400).

Fig 1B: Staining of spleen section with anti-Fx1A (-T) (200 µg/ml). No staining of T-cells around arteriole (arrow) or B-cells in peripheral white pulpa is observed. Only macrophages in red pulpa show endogenous peroxidase activity.(x400).

Fig 1C: Staining of spleen section with Moab F2/8 (anti-GP 90). Staining of T-cells in PALS (arrow) and staining of sinusoid endothelial lining cells (arrowhead) is present. No staining of B-cells in peripheral white pulpa is observed. (x 450).

Fig 1D: Staining of spleen section incubated with Moab F1/12 (anti-GP 330). No staining of T-cells in PALS (arrow) or B-cells in peripheral white pulpa is observed. Only macrophages show endogenous peroxidase activity (x450)

Arrow indicates central arteriole,
Arrowheads indicate sinusoid endothelial lining cells,
pwp= peripheral white pulpa, rp = red pulpa.

Thymus.

The results of the staining of thymus tissue are summarized in table 2, and illustrated in fig 2.

Table 2

	Staining pattern of rat thymustissue in Cortex	Medulla
anti-Fx1A	+	+
anti-Fx1A (-T)	-	-
Heart eluate	+	+
Moab F2/8 (a-GP 90)	+	+
Moab F1/12 (a-GP 330)	-	-
Moab F3/28 "	-	-
Moab F3/237 "	-	-

+ = positive staining - = no staining

Polyclonal anti-Fx1A IgG (fig 2A), heart eluate IgG and monoclonal anti-GP-90 (fig 2 B) show staining of cells in the thymus cortex and medulla. No staining of thymuscells was observed after incubation with either anti-Fx1A (-T) or anti-GP 330 Moabs.(Table 2)

Lymph nodes

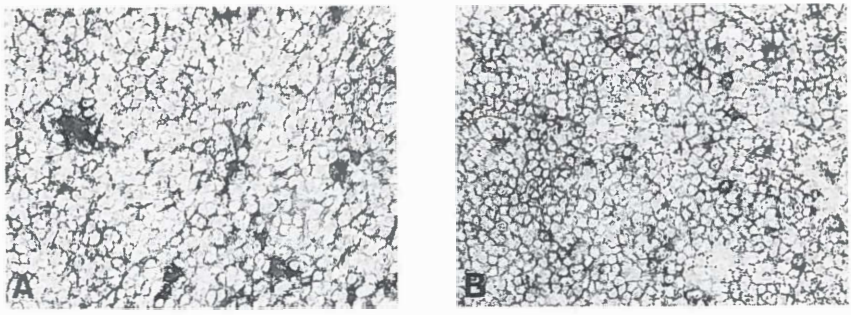
The results of the staining of lymph nodes are summarized in table 3. Polyclonal anti-Fx1A, heart eluate IgG (fig 3A) and monoclonal anti-GP 90 (fig 3B) stain structures in the T-cell area of the lymph nodes.

Table 3

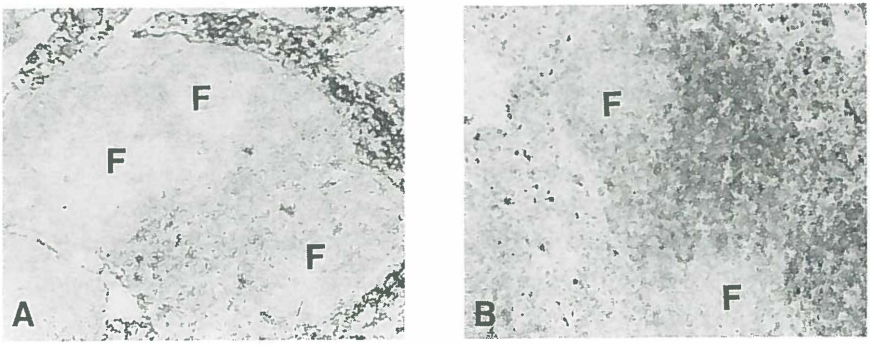
	Staining pattern of rat lymph nodes	
	follicles	paracortex
	B-cell area	T-cell area
anti-Fx1A	-	+
anti-Fx1A (-T)	-	-
Heart eluate	-	+
Moab F2/8 (a-GP 90)	-	+
Moab F1/12 (a-GP 330)	-	-
Moab F3/28 "	-	-
Moab F3/237 "	-	-

+ = positive staining - = no staining

No staining of B-cells was observed. Incubation with anti-Fx1A (-T) did not show staining of lymph node structures just like Moab F1/12 , F3/28 and F3/237 (anti-GP 330).



Legends fig 2. Fig 2A: Thymus sections stained with anti-Fx1A IgG. Staining of all thymocytes is present. Hassels corpsules and capillaries are also stained. (x 650) 2B: Thymus section after staining with Moab F2/8 (anti-GP 90), showing positively stained thymocytes. (x 600)



Legends fig 3. Fig 3A: Lymph node section incubated with heart eluate IgG showing staining of cells in the T-cell area near the follicle. B-cells in follicles are negative. Capillaries between plasma cells outside T/B cell area are also stained. (x 300) Fig 3B: Lymph node section incubated with Moab F2/B (anti-GP 0), showing staining of T-cells near B-cell follicle. B-cells are negative. (x 500)

F = follicle

DISCUSSION

From the present data it is concluded that heterologous anti-Fx1A and rat heart eluate IgG are able to stain T-cell areas of rat lymphoid tissues. In addition, staining patterns of anti-Fx1A and the Moab anti-GP 90 (Moab F2/8) are identical upon spleen, lymph node and thymus sections suggesting that the anti-T moiety of anti-Fx1A is directed to the GP 90 epitope. These results confirm previous data showing anti-GP 90 specificity in rabbit anti-Fx1A using immunoblotting techniques [14].

The anti-RTE α5 antibodies show the same staining pattern as observed with anti-Fx1A IgG upon spleen sections. Since polyclonal anti-GP 330 abs, normal rabbit IgG or rabbit-anti-rat albumin did not stain these T-cell areas or sinusoidal lining cells, these findings indicate the presence of anti-GP 90 antibodies in anti-RTE α5 antiserum. These data are at variance with the data presented by Ronco et al [142], but might be attributed to the different techniques used.

Whether the antibodies, which stain T-cell areas, recognize exclusively T-cell membranes or also reticulumcells localized in these areas is difficult to establish. However incubation of thymus cell suspensions clearly indicated positive membrane fluorescence of thymocytes only using either polyclonal anti-Fx1A IgG [14] or monoclonal anti-GP 90 (results not shown).
Since anti-Fx1A antibodies show staining of endothelial lining cells of the red pulp sinusoids apparently similar epitopes are present on these structures and T-cell areas. A similar staining pattern of sinusoids of the human spleen can also be observed using monoclonal antibodies against human suppressor T-cells [33, 75, 169]. Monoclonal anti rat T- helper and suppressor antibodies however do not stain the endothelium. Therefore, it is concluded that anti-GP 90 antibodies do not recognize rat helper or suppressor T-cells specifically.
The recent observation by Chatelet et al.[43] that GP 90 is also present on rat glomerular epithelial cells, and the widespread distribution of GP 90 on various tissues (including lymphoid tissues) of rat and mouse, in contrast to GP 330 [8, 43, 44, 139], makes it interesting to find out whether GP 90 plays a role in experimental membranous glomerulopathy. Already some studies indicate such a relationship. Firstly, previous ex vivo perfusion experiments showed that the presence of anti-T specificity within rabbit-anti-Fx1A IgG was important for its ability to bind to the rat glomerulus [14]. Secondly, both monoclonal and polyclonal anti-GP 90 antibodies have been shown to localize along the glomerular capillary wall after injection into rats and or mice in vivo [7, 139]. Whether or not anti-GP 90 antibodies contribute to the nephritogenicity of heterologous Heymann serum, these data indicate that besides GP 330-anti-GP 330, other antigen-antibody interactions may be important in the pathogenesis of membranous immune complex formation.

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES.

VI. Glomerular localization of anti-GP 330 and anti-GP 90 antibodies present in passive Heymann serum.

Wilma M Bagchus[1], PhJ Hoedemaeker[2], JFG Slegers[3] & WW Bakker[1].

1) Department of Pathology, University of Groningen, Groningen,
2) Department of Pathology, University of Leiden,
3) Department of Physiology, University of Nijmegen,
The Netherlands.

SUMMARY

Administration into the rat of heterologous antibodies directed against rat tubular brushborder antigens (Fx1A) leads to membranous glomerulopathy (HICN) associated with glomerular immune complexes (Icxs) at the epithelial side of the glomerular capillary wall (GCW). These anti-Fx1A antibodies (Abs), contain two major specificities i.e anti-GP 330 and anti-GP 90. Although GP 330 was claimed to be the pathogenetic antigen in HICN, our previous experiments showed that binding in vitro and after ex vivo perfusion of anti-Fx1A abs was dependent only on the presence of anti-GP 90 abs.
In this study it is shown that the non-localizing properties of anti-GP 330 abs is not a consequence of ischaemia, since experiments using a controlled perfusion system also showed no localization of anti-GP 330 abs, in contrast to anti-GP 90 abs. However, intravenous injection of anti-GP 330 antibodies resulted in granular deposits of rabbit IgG, in contrast to (faint) linear deposits after injection of anti-GP 90 abs. Using alternating and combined perfusion studies with these antibodies, it is clearly shown that anti-GP 90 abs do not influence localization of anti-GP 330 abs in this system. In addition, systemic administration of anti-GP 90 abs combined with perfusion of anti-GP 330 abs do not promote localization of anti-GP 330 abs. Although directly after i.v. injection of anti-Fx1A abs anti-GP 90 abs may localize in the GCW, the data presented exclude a dominant role for

anti-GP 90 abs in the formation of granular Icxs in HICN, and indicate that Icx formation in HICN is due to anti-GP 330 abs.

INTRODUCTION.

Research into the role of mechanisms of immune complex formation in glomerular disease has centered around a few experimental models; experimental serum sickness glomerulonephritis, anti-GBM nephritis and the autologous and heterologous immune complex glomerulonephritis [177] . Autologous immune complex nephritis (AICN) is induced by immunization of rats with a tubular brushborder fraction (Fx1A) leading to the formation of anti-brushborder antibodies (Abs) [3, 63, 70, 77, 89]. Heterologous immune complex nephritis (HICN) in the rat is induced by administration of heterologous antibrushborder Abs [21, 23, 54, 67, 69]. Both models lead to membranous nephropathy associated with glomerular immune complexes (Icxs) at the epithelial side of the glomerular capillary wall (GCW). It has been demonstrated that these immune complexes form in situ [54, 57].

Since Fx1A is a crude preparation, heterologous Abs against Fx1A contain many specificities. At least two major specificities were described previously [14, 19] : one directed to brushborder antigens (anti-GP 330) and one directed to an antigen present on i.e. thymocytes and brushborder (anti-GP 90). In contrast autologous anti-Fx1A Abs only showed anti-GP 330 activity.

In an investigation into the role of these two different specificities in heterologous anti-Fx1A IgG in Icx formation we demonstrated earlier that the binding of heterologous anti-Fx1A Abs in the GCW was dependent on the presence of anti-GP 90 Abs, rather than on the presence of anti-GP 330 Abs [14]. Although anti-GP 90 Abs localize along rat GCW after injection [7], [139], the importance of anti-GP 90 Abs for Icx localization was not supported by other authors who claimed the GP 330 to be the only nephritogenic antigen involved in AICN and HICN [105, 106] .

Therefore additional experiments concerning the role of anti-GP 90 Abs for Icx formation in HICN were performed. First we studied the localization of Icxs after intravenous injection of anti-GP-90 and anti-GP-330 Abs. Secondly, we repeated the perfusion experiments using

anti-GP-330 Abs under controlled conditions [157]. Thirdly the interaction between anti-GP 90 and anti-GP 330 Abs in Icx formation was studied by combined and alternating perfusion into the rat kidney of these Abs, as well as after systemic administration of anti-GP 90 Abs combined with ex vivo perfusion of anti-GP 330 Abs.

MATERIAL AND METHODS

Animals. All experiments were performed in SPF female Wistar rats 8 to 12 weeks of age, varying in body weight from 120 to 140 g. The animals were fed with pellet food (RMBH, Woerden, The Netherlands) and had free access to tap water.

Antibodies. Preparation, isolation and testing of rabbit-anti-FxlA IgG was done as described earlier [67]. The Abs were depleted for anti-T activity by immuno-absorption to rat heart tissue extract, as described previously [14]. Since immunoblotting studies showed that these Abs reacted only with GP 330 ,they are referred to as monospecific rabbit anti-GP 330. IgG was eluted from rat heart tissue used for immuno-absorption, repurified , and with immunoblotting shown to contain Abs against GP 90 as described previously. These Abs are referred to as monospecific rabbit anti-GP 90 IgG.
Comparison of the end point titres of anti-brushborder immunofluorescence on normal kidney sections, indicated that 25 μg/ml rabbit-anti FxlA IgG had the same titre as 100 μg/ml monospecific rabbit anti-GP 330 or 100 μg/ml monospecific rabbit anti-GP 90. Therefore we took a factor 4 to compare the results of rabbit anti-FxlA IgG with monospecific rabbit anti-GP 330 and monospecific rabbit anti-GP 90.
Moab against GP 90 (F2/8) was a kind gift from Dr. P. Verroust, Paris. The characterization and purification of this Moab has been described in previous reports [139, 140].

Immunofluorescence. The presence of rabbit, rat or mouse IgG in rat kidneys was examined using direct immunofluorescence techniques on frozen kidney sections of 4 μ according to standard methods [70] with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit (GAR)

IgG , FITC-conjugated goat anti-mouse (GAM) IgG, or FITC-conjugated rabbit-anti-rat (RARa) IgG (Nordic, Tilburg, The Netherlands). The localization of antibodies in glomeruli was studied by indirect IF. The antibodies either were intravenously (i.v.) injected in intact animals or perfused ex vivo or in isolated kidney under controlled circumstances. In a few experiments i.v. injection and perfusion were combined.

Table 1. Presence of rabbit IgG (R) and /or rat IgG (Ra) in rat kidneys 24 h and 14 days after intravenous injection, as detected by IF using FITC-conjugated GAR IgG or FITC-conjugated RARa IgG.

	ANTIBODY								
	rabbit anti-Fx1A IgG			monospecific rabbit-anti-GP 330 IgG			monospecific rabbit-anti-GP 90 IgG		
Concentration	24 h	14 d		24 h	14 d		24 h	14 d	
	R	R	Ra	R	R	Ra	R	R	Ra
10 µg	+/-	+/-	-	nd	nd	nd	nd	nd	nd
20 µg	+	+	-	+/-	+/-	-	nd	nd	nd
50 µg	+	+	+/-	+	+	-	-	-	-
100 µg	++	++	++	++	++	+	-	-	-
1 mg	nd	nd	nd	nd	nd	nd	-	-	-
2 mg	nd	nd	nd	nd	nd	nd	+/-	nd	nd

- = no staining, +/- = faint staining, + = positive staining, ++ = strong staining , nd = not determined, h= hours, d =days

Intravenous injection. Rabbit-anti-Fx1A IgG, monospecific rabbit anti-GP 330 IgG or monospecific rabbit anti-GP 90 IgG were i.v. injected, in different concentrations as stated in table 1.
After 24 h kidneys of these rats were obtained by nephrectomy under halothane anaesthesia. The remaining kidney was removed after killing the rat 14 days after i.v. injection. Pieces of rat kidney tissue were snap frozen in Freon 12 (-80 C) and the presence of rabbit IgG and/or rat IgG was evaluated by IF.

Perfusion protocols. Abs were perfused into the rat kidney using a standard perfusion method with minor modifications [9, 57]. Under halothane anaesthesia, the relevant ligations were made and a cathether was inserted into the aorta just below the renal artery. To

enable perfused fluid to escape from the kidney a puncture was made in the renal vein. Perfusion of PBS pH 7.3 or IgG (37 C) was done at a rate of 2 ml/min by means of a LKB pump (Bromma, Sweden) until the kidney became pale and no visible blood escaped from the punctured renal vein. Subsequently the perfusion was continued with 8 ml perfusate (2ml/min) as listed hereafter, followed by perfusion with 4 ml PBS pH 7.3 to remove non-bound IgG, before the kidney was removed and processed for immuno-fluorescence microscopy.

In this way we studied the localization of rabbit-anti-Fx1A IgG, monospecific rabbit-anti-GP 330 IgG and monospecific rabbit anti-GP 90 IgG in the concentrations stated in table 2. Furthermore we studied the possible interaction between the different abs in relation to their localization by perfusing a mixture of rabbit-anti-GP 330 (150μ g/ml) together with monoclonal anti-GP 90 Moab, in a concentration of respectively 100 ,150 and 200 μg/ml. In addition in two experiments 2 ml monospecific anti-GP 330 (300 μg/ml) was perfused, followed by perfusion with 2 ml anti-GP 90 Moab (500 μg/ml). This sequence was repeated once. In two experiments identical procedures were used, but the sequence was reversed, i.e we started with anti-GP 90 Moab. Between each perfusate, 2 ml PBS pH 7.3 was perfused to prevent immune complex formation in the glomerular capillaries.

Perfusion experiments under controlled conditions were conducted by Prof.Dr. Slegers at the Department of Physiology of the University of Nijmegen. The perfusion system was described previously [157]. In this system no ischemia occurs. The temperature was kept constant (37.5 C) as well as the renal perfusion flow. A flow of 15.1 ml/min was necessary to obtain a renal perfusion pressure of 105 mg Hg. Generally a period of 30 minutes perfusion at 15 ml/min was allowed for the kidney to stabilize before starting the experimental protocol. To study localization of rabbit IgG, 100 ml recirculating perfusate was used, and perfusion time of IgG was 20 minutes, followed by perfusion with normal medium for 4 minutes to remove unbound IgG. Then pieces of kidneys were snap frozen and processed for IF. The localization of various concentrations of perfused rabbit-anti-Fx1A IgG , monospecific rabbit anti-GP 330 and monospecific rabbit anti-GP 90 IgG was studied using IF (Table 3).

To study if systemic administration of anti-GP 90 Abs might influence the localizing properties of rabbit-anti-GP 330 IgG after ex vivo

perfusion the following experiments were performed. Monospecific rabbit anti-GP 90 (400, 600 and 800 μg respectively) was i.v. injected, and after 4 h, 8 ml monospecific rabbit anti-GP 330 (100 μg/ml) was perfused in the left kidney, followed by perfusion with 4 ml PBS, p H 7.3. The left and right kidney were removed and evaluated for the presence of rabbit IgG by IF. In two experiments, 0,4 ml containing 2 mg Moab anti-GP 90 was i.v. injected . After 4 h, 8 ml monospecific rabbit anti-GP 330 was perfused in the left kidney (100 μg /ml or 250 μg/ml) according to the procedures described above. Both kidneys were removed and evaluated for the presence of mouse and rabbit IgG by IF.

RESULTS

i.v. injection of antibodies.

Injection of rabbit anti-Fx1A IgG into rats results in fine granular staining after 24 h. At day 14 next to rabbit IgG, granular deposits of rat IgG could be observed, depending on the initial concentration of anti-Fx1A IgG used (table 1). A slight increase in the binding of heterologous anti-Fx1A IgG was noted in glomeruli of kidney sections obtained after 24 h compared to kidney sections of the same animal

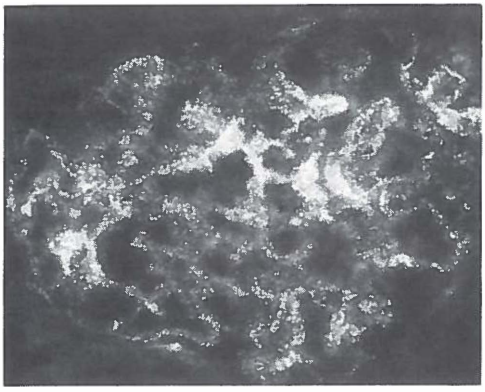


Fig.1: Glomerulus of a rat kidney 14 days after injection of monospecific anti-GP 330 IgG (100 μg/ml), showing granular deposits of rabbit IgG typical of Heymann nephritis. Staining with FITC-conjugated GAR IgG. (x 400)

obtained after 14 days. The results of i.v. injection of different amounts of monospecific rabbit anti-GP 330 IgG are summarized in table 1. Fig. 1 shows an example of the typical granular pattern of rabbit

IgG along the GCW as observed by IF 14 days after i.v. injection. In general, the pattern is the same as observed after injection of rabbit anti-Fx1A IgG. Intravenous injection of comparable concentrations of monospecific rabbit anti-GP 90 IgG did not result in localization of rabbit IgG in the GBM (table 1). Only using high concentrations (2 mg) some faint linear fluorescence could be observed after 24 hrs.

Table 2
Presence of rabbit IgG following perfusion in the rat kidney detected by IF using FITC-conjugated GAR IgG.

	ANTIBODY		
	Rabbit anti-Fx1A IgG	monospecific rabbit anti-GP 330 IgG	monospecific rabbit anti-GP 90 IgG
Concentration µg/ml			
10	+/-	nd	nd
20	+	-	nd
50	++	-	+
100	++	-	++
150	++	-	++

- = no staining, +/- = faint staining, + = positive staining, ++ = strong staining, nd = not determined

Table 3: Presence of rabbit IgG following perfusion in the controlled kidney perfusion system, detected with IF using FITC-conjugated GAR IgG

	ANTIBODY		
	Rabbit anti-Fx1A IgG	monospecific rabbit anti-GP 330 IgG	monospecific rabbit anti-GP 90 IgG
Concentration µg/ml			
1.5	+/-	nd	nd
3	+	nd	nd
6	+	nd	+/-
10	++	nd	nd
25	++	--	nd

- = no staining, +/- = faint staining, + = positive staining, ++ = strong staining, nd = not determined

Perfusion studies.

The results of the perfusion of different amounts of rabbit-anti-Fx1A IgG, monospecific rabbit-anti-GP 330 IgG and monospecific rabbit-anti-GP 90 IgG are summarized in table 2. The IF pattern observed after perfusion of rabbit-anti-Fx1A IgG and monospecific rabbit-anti-GP 90 IgG was identical. No localization of rabbit IgG was observed after perfusion of monospecific rabbit-anti-GP 330 IgG. The results of the perfusion experiments under controlled conditions are summarized in table 3. The results obtained in both perfusion systems were similar. Fig. 2 shows an IF photograph of a glomerulus after controlled perfusion with 100 ml of 6 μg/ml rabbit-anti-Fx1A IgG, Fig.3 after perfusion with 6 μg/ml monospecific rabbit-anti-GP 90.

Perfusion of mixtures of monospecific anti-GP 330 IgG and Moab anti-GP 90 or alternating perfusion with these antibodies did not result in localization of rabbit IgG in the rat glomerulus. In contrast, in all experiments mouse IgG was readily detected in the rat kidney along the GCW .

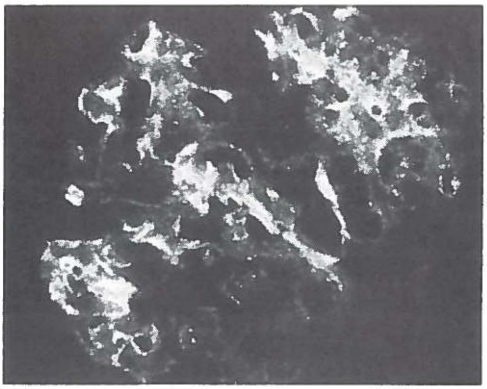


Fig.2: IF photograph of a glomerulus of a rat kidney after controlled perfusion with 100 ml of 6 μg/ml rabbit-anti-Fx1A IgG and incubated with FITC-conjugated GAR IgG (x 400), showing strong staining along the GBM.(x400)

An example of a glomerulus positive for mouse IgG is shown in Fig. 4. In the alternating perfusion experiments starting with either monospecific rabbit anti-GP 330 or anti-GP 90 Moab only binding of anti-GP 90 Moab was observed.

Fig.3: IF photograph of a glomerulus of a rat kidney perfused with monospecific anti-GP 90 IgG in the controlled perfusion system, and incubated with FITC-conjugated GAR IgG. Staining along the GCW is observed. (x400)

Fig.4: Glomerulus of a rat kidney perfused with Moab anti-GP 90, (8 ml, 150 µg/ml) and incubated with FITC-conjugated GAM IgG, showing staining along the GCW. (x 400)

Fig.5: Glomerulus of the right kidney of a rat after injection of Moab anti-GP 90 and stained with FITC-conjugated GAM Ig, showing staining along the GCW and also (faint) staining of the brushborder of the proximal tubules. (x 400)

Combined intravenous injection and perfusion.

IF of the right kidney of rats injected with monospecific rabbit anti-GP 90 did not show the presence of rabbit IgG. Subsequent perfusion of the left kidney 4 hrs later with monospecific rabbit anti-GP 330 IgG did not show the presence of rabbit IgG in these perfused kidneys.

Intravenous injection of 2 mg of Moab anti-GP 90 resulted in the presence of mouse IgG in both the right and the left kidney (Fig.5), as observed with IF after 4 hrs. In addition mouse IgG was observed along the brushborder of a few proximal tubules. After perfusion of the left kidney of these animals with monospecific anti-GP 330 no localization of rabbit IgG could be observed.

DISCUSSION

To elucidate the role of anti-GP 90 abs of Heymann nephritis, we performed both intravenous injection and perfusion experiments. From controlled perfusion of the isolated rat kidney it appeared that heamodynamic factors do not play a major role in the localization of anti-FxlA abs, since the results were similar in both perfusion systems. This was previously observed by Fleuren et al. [71], using the same perfusion systems.

In the controlled ex vivo perfusion system as well as in experiments described previously [14] , rabbit anti-FxlA IgG and monospecific anti-GP 90 IgG readily localize along the GCW, in contrast to monospecific anti-GP 330 IgG. In contrast i.v. injection of rabbit-anti-FxlA IgG or monospecific rabbit anti-GP 330 IgG result in granular deposits of rabbit IgG after 24 hrs and 14 days. Monospecific rabbit anti-GP 90 IgG in comparable concentrations does not localize after i.v. injection, although much higher amounts induce a faint linear staining , in contrast to the granular staining observed after i.v. injection of monoclonal anti-GP 90 abs.

Localization of anti-GP 90 antibodies in vivo has been found by other authors as well [7, 139]. This can be explained by the finding that the GP 90 epitope is present on many rat tissues [7, 43], including glomerular endothelial and epithelial cells, [44, 139] in contrast to GP 330 which is confined to epithelial cells [44, 59]. Glomerular localization of anti-GP 90 Abs in vivo seems dependent on the amount of Abs used, since most of the injected abs are deposited in other organs [139] . The strong staining observed after i.v. injection of Moab anti-GP 90 (Fig.5) compared to the very faint staining after i.v. injection of monospecific rabbit anti-GP 90 Abs might be explained by the different concentration of specific antibodies in the different

batches. Indeed it is true that monoclonal anti-GP 90 Abs posses a much higher titre of anti-brushborder Abs compared with monospecific anti-GP 90 Abs as evaluated on rat kidney sections (not shown).
A possible interaction between anti-GP 90 and anti-GP 330 abs which might explain altered localization of anti-GP 330 abs in vivo versus ex vivo was excluded by the data obtained from perfusion of mixtures of Moab anti-GP 90 and monospecific rabbit anti-GP 330 IgG and alternating perfusion studies with these Abs. Though localization of anti-GP 90 Moab was clearly seen, the presence of rabbit anti-GP 330 could not be observed.
To study whether systemic anti-GP 90 Abs per se might influence the localization of anti-GP 330 Abs, we i.v. injected monospecific rabbit-anti-GP 90 in nonlocalizing concentrations, as well as mouse anti-GP 90 Moabs. Intravenous injection of monospecific rabbit-anti-GP 90 and subsequent ex vivo perfusion of the left kidneys of these animals with monospecific rabbit anti-GP 330 IgG did not result in localization of rabbit IgG. Intravenous injection of anti-GP 90 Moab and subsequent perfusion of the left kidney with monospecific anti-GP 330, resulted in glomerular deposits of anti-GP 90 Moab only and increased glomerular permeability, concluded from the presence of mouse IgG in the brushborder of the rat tubuli. This indicates that neither anti-GP 90 Abs per se nor increased permeability of the GCW influence localization of anti-GP 330 Abs.
Early localization of heterologous anti-Fx1A in HICN is known to be more linear than granular [54, 57]. In addition Jeraj et al. [99] showed binding of heterologous anti-Fx1A to endothelial cell membranes. Since the linear pattern of binding of anti-Fx1A resembles the binding of anti-GP 90 to the GCW and since it was shown that endothelial cells have the GP 90 epitope on their cell membranes, localization of IgG after perfusion with heterologous rabbit-anti-Fx1A seems to be caused by anti-GP 90 antibodies.
There is a striking difference in the localization of monospecific rabbit anti-GP 330 IgG after i.v. injection and ex vivo perfusion. In both perfusion models no localization of rabbit anti-GP 330 was observed , in contrast to the clear localization after i.v. injection. This might be explained by the different accessabilities of anti-GP 330 or by the distribution of GP 330 epitopes on the epithelial cell (8). It is possible that using longer perfusion times of over 60

minutes and with keeping fysiological vitality of the kidney intact, aggregates might originate. Though these experiments have been performed by Couser et al. [54] using anti-Fx1A, and granular subepithelial deposits were not seen before 2 hrs, the presence of anti-GP 90 activity in sheep-anti-Fx1A IgG make these data inconclusive.

The present data and the absence of perfusion studies using well defined anti-GP 330 antibodies warrant the question whether in AICN in situ localization plays a role, since only GP 330 is claimed to be the pathogenetic antigen in this model. Although localization of autologous anti-brushborder abs has been described [71, 92, 114, 131, 147], it is possible that these fractions possessed not only anti-GP 330 activity. The recent observation of Kamata et al. [104] that eluates of AICN rats with proteinuria posses an anti-GP 90 like component (e.g GP 95) indicate that this specificity might be responsible for the depositions observed. Perfusion studies using eluates of rats with AICN with and without proteinuria (i.e with and without anti-GP 95) might elucidate this question.

Another explanation is that granular deposits of anti-GP 330 antibodies on glomerular epithelial cells only form after crosslinking of these bound antibodies, followed by redistribution,`capping' and `shedding' of the immune complexes. This was recently shown on cultured epithelial cells using heterologous anti-GP 330 abs [35]. The in vivo data in which redistribution and shedding of GP 330 anti-GP 330 complexes of the epithelial cell was prevented by chlorpromazine and the absence of granular subepithelial deposits indicates that the same mechanism might operate in vivo [36].

In this study it is clearly shown that the non-localizing properties of monospecific rabbit anti-GP 330 IgG is not a consequence of ischaemic alterations, known to occur in the non-controlled perfusion system. In addition it is shown that anti-GP 90 antibodies do not influence the non-localizing properties of anti-GP 330 abs in the perfusion system. Although, directly after i.v. injection of heterologous anti-Fx1A abs anti-GP 90 abs probably localize in the GCW, the in vivo data using monospecific anti-GP 330 abs exclude a dominant role for anti-GP 90 abs in granular Icx formation in HIC.

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES. II. IMMUNE COMPLEX GLOMERULOPATHY IN RATS INDUCED BY HETEROLOGOUS ANTITHYMOCYTE SERUM.

WM Bagchus, J Donga, PhJ Hoedemaeker and WW Bakker
University of Groningen, 9713 EZ Groningen, The Netherlands, and
University of Leiden, Laboratory of Pathology, 2333 AL Leiden,
The Netherlands.

SUMMARY

Injection of rabbit anti-rat thymocyte serum (ATS) i.v. into rats induces a transient glomerulopathy characterized by immune aggregates localized in the mesangium and along the glomerular capillary wall, as detected by immunofluorescence (IF) techniques. Neither light microscopical alterations in the kidney, nor proteinuria could be detected in these animals and no autologous IgG could be observed in the glomeruli during the observation period (45 days). Results from ex vivo perfusion studies showed identical localization of immune aggregates which, in electron microscopy, appear to be localized subepithelially. The ATS used, was monospecific in that no other specificities could be detected after thorough absorption with rat tissue extracts including tubular brushborder antigens, so it is concluded from these data that ATS is able to participate in the formation of immune complexes in situ by recognizing epitopes both in the mesangium and at the epithelial side of the glomerular basement membrane.

INTRODUCTION

A prominent feature of membranous glomerulopathy is the formation of immune complexes along the glomerular capillary wall leading to glomerular injury and proteinuria [177]. Several experimental models of this disease have been described in the rat using immunization with

homologous tubular brushborder antigens (BBag) or using injection with heterologous antibodies against tubular brushborder antigens (aBBag) [21, 67, 69, 70, 89]. The latter model, denoted as heterologous immune complex glomerulopathy (HICG), is characterized by immediate formation of immune aggregates of rabbit IgG along the epithelial side of the glomerular basement membrane (GBM) following injection. The immune aggregates form in situ in the GBM rather than being deposited from the circulation [54, 57]. Ten days after injection of the heterologous antibody an autologous phase starts, indicated by the presence of autologous IgG in immune deposits along the capillary walls [16, 67]. In a previous study we have shown that antithymocyte antibodies are present in heterologous aBBag whereas commercially obtained rabbit antirat thymocyte serum (ATS) contains aBBag specificity. In addition, it was shown by absorption studies that anti-T-cell activity in aBBag serum was mediated by specific anti-T-cell antibodies rather than by aBBag cross-reactive with thymocyte membrane antigens [18, 19]. The question was raised whether this anti-T-cell specificity per se possessed nephritogenicity, and we therefore decided to study the contribution of anti-T-cell specificity in the nephritogenicity of ATS. In this article, results of i.v. administration of monospecific ATS, as well as ex vivo perfusion of ATS or control antibodies into the rat kidney, are described. It is shown that, in contrast to normal rabbit IgG (NR IgG) or rabbit antirat albumin (R-a Alb) IgG, i.v. administration of ATS results in a transient glomerulopathy characterized by preponderantly mesangial localization of immune aggregates, and also by localization of immune aggregates along the capillary walls. No significant light microscopical alterations or proteinuria were observed. Subepithelial localization of immune complexes could also be detected following ex vivo perfusion of ATS into the renal artery of the blood free rat kidney, so it is suggested that antigens present in the GBM of the rat kidney are recognized by ATS. In view of the fact that some batches of human ATS are reported to be nephritogenic [34, 161], further study into possible antigenic determinants shared between glomeruli and T cells might be worthwhile.

MATERIALS AND METHODS

Animals. Female outbred Wistar rats 3 months of age were used throughout the study. The rats were fed with RMHB (Woerden, The Netherlands) and received water ad libitum.

Preparation of tissue extracts. Insoluble BBag was prepared from Wistar rat kidneys according to the methods described by Edgington et al. [65]. Rat thymocyte membrane extract and rat heart tissue were prepared as described elsewhere [19]. The extracts were used for absorption purposes.

Preparation of antibodies. Rabbit ATS was raised in Chinchilla rabbits according to Kreeftenberg's method [108], with minor modifications, and 0.2 ml containing 10^8 Wistar thymocytes, isolated as described elsewhere [19], was injected i.v.into female rabbits. On day 5, 0.1 ml bacillus calmette-guerin BCG vaccine (National Institute of Health; Bilthoven, The Netherlands) in 0.5 ml saline was given i.v., and booster injections of cells were administered i.v. on day 14, day 21, and subsequently every 4 weeks. Venous blood (20-40 ml) was collected 30 days after the first immunization and subsequently every month. In some experiments ATS from Natucon was used for comparison. Either heat-inactivated (45 min at 56 C) full serum or 50% ammonium-sulphate-precipitated globulin fractions were used, following routine absorption with fresh rat erythrocytes and with lyophilized rat heart tissue extract according to standard procedures [19] as well as thorough absorption with BBag. Antithymocyte activity was tested after the absorption upon rat thymocytes using a complement-dependent cytotoxicity assay according to Okumura et al [135], with minor modifications [19]. R-aAlb serum was prepared by immunization of Chinchilla rabbits with rat albumin (Sigma) according to the immunization schedule described by Feenstra et al. [67].
The presence of specific antibody was tested using standard double diffusion assays in agar gel. Both R-aAlb and NR IgG obtained from normal rabbits were heat-inactivated, thoroughly absorbed with tissue extracts, fractionated as described for ATS, and tested for nonspecific staining of rat kidney tissue using immunofluorescence (IF) techniques, and for antithymocyte activity using a cytotoxicity

assay. After absorption, ammonium sulphate precipitate fractions of ATS and control sera were adjusted to a final concentration of 10 mg IgG/ml, before use, by Amicon filter concentration (B15 Amicon).

Absorption procedures. Diluted ATS or control sera (1:20) were absorbed with lyophilized tissue antigens (200-300 mg of dry tissue extract per milliliter of antiserum), as described elsewhere [19]. Briefly: the immunoabsorbent was mixed with antibody solution, first for 2 h at room temperature, and subsequently overnight at 4 C. The immunoabsorbent was spun down at 30.000 x g for 60 min.

Experimental procedure. Of ATS, 1 ml, was injected i.v. in the tail vein of the animals, and a second injection of 0.5 ml was given 2 days later. Groups of 5 animals were killed under ether anesthesia after 2 h, 24 h, and 5, 14, 21, and 45 days after the first injection. Control animals received nonimmune NR IgG or R-aAlb antibodies, according to the same schedule.

Ex vivo perfusion. Ex vivo perfusion of normal female Wistar rat kidneys was done as described by Van Damme et al. [57], with minor modifications. Animals were anesthetized using halothane N20, and the left kidney was mobilized. After ligation of the tributaries, the aorta was clipped proximally to the left renal artery and above the bifurcation. The renal vein was also clipped, and subsequently a catheter was inserted into the aorta up to the left renal artery. The catheter was fixed in a ligature. The renal vein was punctured to allow escape of blood and perfused fluids. The kidney was perfused with phosphate-buffered saline (PBS), pH 7.2, at 37 C (2 ml/min) until a pale color was achieved and no visible blood escaped from the punctured renal vein. Subsequently 20 ml ATS or control sera, containing 1 mg/ml IgG, was perfused during 10 min, and this was followed by perfusion with 10 ml PBS at 37 C, pH 7.2, to remove unbound antibodies or complexes, or both. After this period the kidney was taken out and the animal was killed by means of an overdose of halothane. Specimens of the perfused kidneys were prepared for light and fluorescent microscopy, as well as for electron microscopy.

Proteinuria. Urinary protein excretion of experimental animals was measured daily for 14 days, and subsequently every week using the biuret method.

Light microscopy. Pieces of kidney were fixed with Tellyesniszky solution and processed according to standard methods, 2 μ sections were stained with haematoxylin and eosin, periodic acid Shiff, and silver-methamine.

Immunofluorescence microscopy. Small pieces of kidney tissue were snap-frozen in precooled freon (-80 C). Cryostat sections of 2 μ were cut, and - after 2 washings in PBS - direct immunofluorescence was performed by incubating the sections for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (GAR) IgG (Nordic; Tilburg, The Netherlands), FITC-conjugated rabbit anti-rat (RARa) IgG, or FITC-conjugated rabbit anti-rat complement (RAC). After 2 washings with PBS the sections were embedded in mounting medium (Gurr, pH 8.0) and examined under a Leitz Orthoplan fluorescence microscope with Ploemopak illumination. To study staining patterns of ATS or control sera, an indirect immunofluorescence technique was carried out on cryostat sections of normal rat kidney sections. Incubation with ATS was preceded and followed by a wash in PBS. Subsequently, the sections were incubated with FITC-GAR and processed as described for the direct immunofluorescence method.

Electron microscopy (EM). For electron microscopy the processing of the material was done according to the method of Feenstra [67]. Briefly: small pieces of kidney cortex (1 mm3) were fixed in 2% glutaraldehyde for 24 h at 4 C. After a rinse in Tris (Tris M.A.) (hydroxymethyl) aminomethane, maleic acid buffer, pH 7.3, for 2 days at 4 C, the samples were postfixed in 0.1 M phosphate buffered OsO4 for 4 h at 4 C. Further processing of the material was done according to standard methods [91] and stained with 5% uranyl acetate and 3% lead citrate.

Immunoelectron microscopy (IEM). Small pieces of perfused kidney cortex were rinsed in Tris M.A., pH 7.3, for 6 h at 4 C and snap-frozen in freon-12 at -80 C. Cryostat sections (20 μm) were incubated

with peroxidase conjugated GAR IgG (Miles-Yeda) for 18 h at 4 C, and subsequently rinsed at room temperature for 30 min with PBS, pH 7.2. The second incubation with 3.3 diaminobenzidine tetrahydrochloride in a concentration of 0.1%, containing 0.001% H202, was performed for 60 min at room temperature and was followed by a rinse with for 30 min and by postfixation on 0.1 M phosphate-buffered OsO4, for 45 min at room temperature. The sections were embedded in Epon and processed according to standard methods [91].

RESULTS

Specificity of antisera.

After absorption of ATS with normal rat erythrocytes, rat heart disease extract, and BBag, 100% kill at 1/64 dilution and 50% kill at 1/256 dilution using the cytotoxicity assay [19] were observed. This antithymocyte activity was abolished completely after a single absorption of ATS with thymocyte membrane extract. R-aAlb and NR IgG did not show antithymocyte activity when tested for cytotoxicity. Neither the R-aAlb or NR IgG, nor the absorbed ATS, stained brushborders of rat kidney tissue or other rat tissues, such as uterus or liver, when tested in an indirect IF assay; in contrast to ATS, the control antibodies, R-aAlb and NR IgG, did not stain glomerular structures. Glomerular staining was also negative when ATS previously absorbed with thymocyte membrane extracts was used.

Fluorescence microscopy.

Normal rat kidney sections incubated with ATS and subsequently stained with FITC-GAR showed a preponderant mesangial and also granular staining along the capillary walls, as can be seen in Fig. 1. Heterologous IgG containing immune deposits could be demonstrated by direct IF in the glomeruli from kidneys of ATS-treated animals. As can be seen from Fig. 2, again a preponderant staining of the mesangial area could be detected, whereas some granular staining along the capillary walls was also present. After day 14 a gradual decrease of the staining occurred that was no longer detectable at day 45. No autologous IgG or rat complement could be observed in the immune deposits of ATS-treated rats.

Fig. 1. Indirect immunofluorescence of normal rat kidney following incubation with ATS. A preponderantly mesangial staining is observed. x400.

Fig. 2. Direct immunofluorescence of a kidney of a rat injected 5 days earlier with ATS. A granular deposition of rabbit antibody is seen mostly in the mesangium. x400.

After perfusion of ATS into the renal artery of the anesthetized rat ex vivo, a mostly mesangial staining by FITC-labeled GAR was present, but again some granular deposits along the capillary walls could be detected. After i.v. injection or perfusion with control antisera, no staining of heterologous antibody could be seen using FITC-conjugated antibodies.

Electron microscopy.

Electron microscopy examination of kidneys from rats after i.v. injection of ATS or perfusion with ATS showed small subepithelial deposits (Fig. 3). No electron-dense deposits were seen in the mesangium.

These subepithelial deposits were absent in kidneys from rats injected or perfused with NR IgG or R-aAlb. In the subepithelial aggregates of the ATS-treated rats or ATS-perfused kidneys, rabbit IgG could be detected using peroxidase-labeled antibody (Fig. 4), whereas again no significant staining was detected in the mesangium of these kidneys. In contrast to the pictures seen in control animals, kidneys from

Fig. 3. Electronmicrograph of a glomerular capillary wall from the same rat as the one shown in Fig. 2. Electron-dense deposits (arrows) are seen at the subepithelial side of the GBM. x21,341.

animals injected with ATS, or kidneys perfused with ATS, often showed effacement of epithelial foot processes (Figs. 3 and 4).

Light microscopy and proteinuria.

None of the rats treated with ATS or control sera showed proteinuria, whereas the kidneys of these animals, killed at various times, did not show any significant light microscopical alterations. Also, kidneys of rats perfused with ATS or control sera did not show light microscopical alterations.

Fig. 4. Immunoelectronmicrograph of a glomerular capillary wall after perfusion with ATS. Note the electron-dense reaction product indicating the presence of rabbit IgG at the subepithelial side of the GBM. x29,872.

DISCUSSION

Heterologous ATS injected i.v. into normal rats can localize in the kidney for several weeks without inducing light microscopical lesions or proteinuria. Staining of kidney sections from ATS-treated rats for the presence of heterologous IgG using IF shows a mostly mesangial pattern, although to a lesser extent granular staining along the capillary walls can be observed also (Fig. 2). This staining pattern can also be achieved by incubation of normal rat kidney sections with ATS (Fig. 1), so it seems likely that ATS recognizes antigens present in mesangium and in GBM, as was suggested by Harada et al. [81], using anti-Thy-1 serum. Because, in addition, perfusion of ATS in the blood-free rat kidney shows identical results, it is concluded that in situ binding of ATS, rather than trapping of immune complexes, plays a role in the generation of this condition. Although IF shows the presence of ATS bound to the mesangium and to the capillary walls, conventional EM and IEM were only able to identify the ATS bound to the capillary wall.

No electron-dense deposits or staining for heterologous IgG could be detected in the mesangial area. The reason for this discrepancy between light microscopic and the ultrastructural level is not clear. A similar discrepancy is found in cases of human IgM nephropathy [86]; it is probably related to the fact that small and scarce electron-dense deposits are easily missed using electron microscopy.

Although similar amounts of ATS (based on optical density measurements at 280 nm) were i.v. injected or perfused into the kidney, it was observed that the fluorescent staining in the perfused kidneys was more prominent than in the kidneys of i.v. injected rats. This probably is due to the fact that perfusion of ATS into the kidney enables a considerable amount of antibody to pass through the glomeruli, in contrast to i.v. injected ATS, much of which appeared to be trapped in the liver (data not shown).

In our animals, mesangial localization of antibodies after administration of ATS persisted for several weeks - however, no autologous phase could be detected, in contrast to the heterologous mesangiopathy in the rat described by Seelig et al. [153]. The reason for the absence of an autologous phase in the ATS-treated rats remains unclear, and it may be related to the direct immunosuppressive

capacity of ATS or to the generation of suppressor cells, as described by Abdou [1] in humans after ATS administration. Alternatively, in other glomerulopathies, even when readily detectable heterologous antibodies are present in sufficient amounts for several weeks, autologous antibody deposition might be undetectable [2].

The question arises what epitopes, shared by glomerular tissue and thymocyte membranes, are recognized by ATS. The ATS used in this study is of polyclonal origin, so it cannot be excluded that different epitopes in the mesangium and GBM are recognized by this antiserum. On the other hand, the thoroughly absorbed ATS used in this study may be considered to be monospecific - reflected, for instance, by the fact that no staining of other rat tissues, including BBag, could be detected. Therefore, we think that the results might be explained by recognition of a shared epitope present in GBM, in mesangium and on thymocytes rather than by detection of different epitopes in these tissues because of crossreactivity of the ATS used. Thy-1 antigens are thought to be present in mesangium and GBM [81, 97, 123], so this antigen seems a likely candidate to be recognized by ATS leading to the staining pattern observed. Recognition of this ATS of rat major histocompatibility complex determinants, such as Ia antigens expressed on a small subset of glomerular cells [151], seems unlikely because, as observed by others as well [83], we were not able to show any glomerular staining using monoclonal antirat Ia antibody.

Taking the results together, localization of ATS in vivo and in vitro reflects in situ recognition of thymocyte membrane epitopes that are also present in mesangium and in the lamina rara externa of the GBM. Irrespective of the fact that these antibodies do not give rise to proteinuria, they localize in the glomeruli for several weeks, so rabbit ATS must be considered nephritogenic in this model. This observation might explain the nephritogenicity of some batches of antihuman thymocyte serum used in the clinical situation [34, 161].

THE SPECIFICITY OF NEPHRITOGENIC ANTIBODIES.
IV. BINDING OF MONOCLONAL ANTI-THYMOCYTE ANTIBODIES TO RAT KIDNEY.

W.M. Bagchus, J. Donga, J. Rozing, Ph.J. Hoedemaeker (1) and W.W. Bakker (2).

Department of Pathology, University of Groningen, Groningen.
(1) Department of Internal Medicine, Erasmus University, Rotterdam
(2) Department of Pathology, University of Leiden, Leiden.
The Netherlands.

SUMMARY

Polyclonal rabbit antirat thymocyte serum (ATS) has been shown to form in situ glomerular immune aggregates following perfusion into normal rat kidney ex vivo. This may be due to the presence of T-cell-like epitopes in the rat kidney, or it may be a result of contaminating anti-connective-tissue antibodies in ATS. To exclude the latter possibility we investigated binding to the rat kidney of three different (mouse) monoclonal antirat thymocyte antibodies (anti-T-cell MoAbs), directed to Thy 1.1 antigen, as well as (control) anti-B cell MoAbs. The MoAbs were incubated in vitro with kidney sections or perfused into the blood free rat kidney ex vivo. It was shown using immunofluorescence (IF) and immunoelectron microscopy (IEM) (peroxidase technique) that the anti-T-cell MoAbs used, in contrast to anti-B-cell MoAbs, are able to bind with glomerular capillary walls, and with mesangial structures after incubation in vitro or perfusion ex vivo. Although staining patterns are not completely identical, the reaction product is clearly demonstrated throughout the glomerular basement membrane (GBM) and along the plasma membranes of endothelial and epithelial cells, after contact with either of the three anti-T-cell MoAbs used. It is concluded that the presence of T-cell-like epitopes in the rat kidney may lead to immune complex formation following contact with anti-T-cell MoAbs. The nephritogenicity of rabbit ATS as well as of some batches of clinically used ATS, may also

be explained by this mechanism rather than by the usually assumed presence of contaminating antibodies in these polyclonal antisera.

INTRODUCTION

Anti-human-thymocyte serum (a-human ATS) is used as an immunosuppressive agent in transplantation [34],[161). The presence of nephritogenic antibodies in batches of this antiserum has been reported [34, 161]. Contaminating stromal elements in the inoculum used for immunization are thought to be responsible for the presence of these undesired antibodies [120].

Rabbits immunized with thoroughly washed rat thymus cell suspensions also produce nephritogenic antibodies (ATS)[19]. Administration of these antibodies into rats i.v. causes a transient glomerulopathy, characterized by immune aggregates localized in the kidney--mostly in the mesangium, and also along the glomerular basement membrane (GBM) [9]. The possibility exists that the nephritogenicity of ATS is due to epitopes shared among T-cell membranes and kidney structures, such as the Thy 1.1 antigen, which is present on rat thymocyte membranes and mesangium cells of the rat kidney [81, 97, 123, 136].

Interestingly, rabbit antibodies directed to brushborder antigens--which, after injection into rats, induce heterologous immune complex glomerulopathy-- also contain anti-T-cell specificity [18, 19, 57]. In earlier studies we were able to demonstrate that this anti-T-cell activity is crucial for localization along the GBM after perfusion ex vivo [10, 14].

In order to characterize the epitopes presumably shared between thymocytes and kidney, we studied the renal binding of (mouse) monoclonal antibodies directed to thymocytes using immunofluorescence (IF) and immunoelectron microscopy (IEM). The binding was studied following incubation of kidney sections with the antibodies as well as after perfusion of these MoAbs in the normal rat kidney ex vivo. The results show that monoclonal anti-T-cell antibodies, in contrast to monoclonal antibodies directed to rat B cells, are able to bind in mesangial matrix as well as in the GBM after ex vivo perfusion or incubation in vitro with rat kidney tissue. It is concluded that in glomerular mesangium as well as in GBM, epitopes occur that are

recognized by anti-T-cell paratopes. It is likely, therefore, that the nephritogenicity of polyclonal ATS observed previously [9]-- as well as that of some batches of a-human ATS prepared for clinical use--is due to the presence of T-cell epitopes in the glomerulus rather than to anti-kidney antibodies induced by contaminating connective tissue in the inoculum used for preparation of these antisera.

MATERIALS AND METHODS.

Animals. Female outbred Wistar rats of three months of age were used throughout the study. The rats were fed with RMBH (Woerden, The Netherlands) and received water ad libitum.

Monoclonal antibodies (MoAbs). Three mouse MoAbs against rat thymocytes and one against rat B cells were used in this study. Ascites-derived MoAbs were prepared using 50% saturated ammonium sulphate precipitation, according to standard techniques. After dialysis against phosphate-buffered saline (PBS), pH 7.3 for 72 h, optical density was measured at 280 nM by spectrophotometry, and the antibodies were stored at -20C at a concentration of 1 mg/ml. Some characteristics of these antibodies are summarized in table 1.

Ex vivo perfusion of normal rat kidney with monoclonal antibodies. Ex vivo perfusion of normal female Wistar rats was done according to van Damme [57] with minor modifications as described elsewhere [9]. After kidney perfusion with PBS, pH 7.2, 37 C (2ml/min), 10 ml MoAb containing 100 μg IgG/ml PBS was perfused. After perfusion of MoAb, subsequently 5 ml PBS was perfused to remove nonfixed antibodies and/or complexes. A small piece of kidney tissue was removed from the lower pole of the kidney, snap frozen in freon 12, and stored at -80 C for IF microscopy. Kidneys were subsequently perfused for IEM with 20 ml of 0,2% freshly prepared glutaraldehyde, followed by perfusion with 5 ml Tris (hydroxymethyl) aminomethane (Tris) maleic acid, pH 7.3 (Tris MA). The kidney was taken out, and small pieces of kidney were rinsed in Tris MA for 2 h at 4C, snap frozen in freon 12 at -80C and processed as described below.

Table 1 Monoclonal antibodies used for in vitro incubation and ex vivo perfusion of normal rat kidney.

1) Code	Subclass	2) Antigenic determinant	3) Distribution
ER 14	IgG 1	25 kD	96% thymocytes 3% spleen cells 0% peripheral T lymphocytes 31% bone marrow cells
ER 4	IgG 2a	25 kD	98% thymocytes 1% spleen cells 5% peripheral T lymphocytes 20% bone marrow
ER 5	IgG 3	25 kD	97% thymocytes 15% spleen cells 27% bone marrow cells
His 11	IgG 1	-	B cell areas of the rat spleen

1) The ER-coded antibodies were prepared by Dr.J.Rozing, Erasmus University, Rotterdam; MoAb HIS 11 was a gift from Prof.Dr.P. Nieuwenhuis, Department of Histology, Groningen.
2) Molecular weight of antigenic determinants, as detected by immunoprecipitation is indicated in kilodaltons (kD) [144].
3) Tissue distribution of the antibodies was determined by FACS analysis with fluorescein-isothiocyanate (FITC)-conjugated IgG [144]. The FACS data were confirmed with immunohistology. The tissue distribution of His 11 [109] was detected by immunohistology (peroxidase method).

Immunofluorescence microscopy (IF). To study binding of MoAb with normal rat kidney, in vitro cryostat sections (4 μm) of normal kidney tissue were washed twice in PBS followed by incubation with 0.05 ml MoAb (50 μg/ml) for 30 min, washed twice, and subsequently incubated with 0.05 ml fluorescein-isothiocyanate (FITC)-conjugated goat antimouse IgG (GAM) (1/40, Nordic, Tilburg, The Netherlands) for 30 min. After two washings in PBS the sections were embedded in mounting medium (Gurr, pH 8.0) and examined under a Leitz Orthoplan fluorescence microscope with Ploemopak illumination. Localization of mouse IgG, after perfusion of MoAb into normal rat kidney, was studied by staining of 4 μm cryostat sections with FITC-conjugated GAM, as described above.

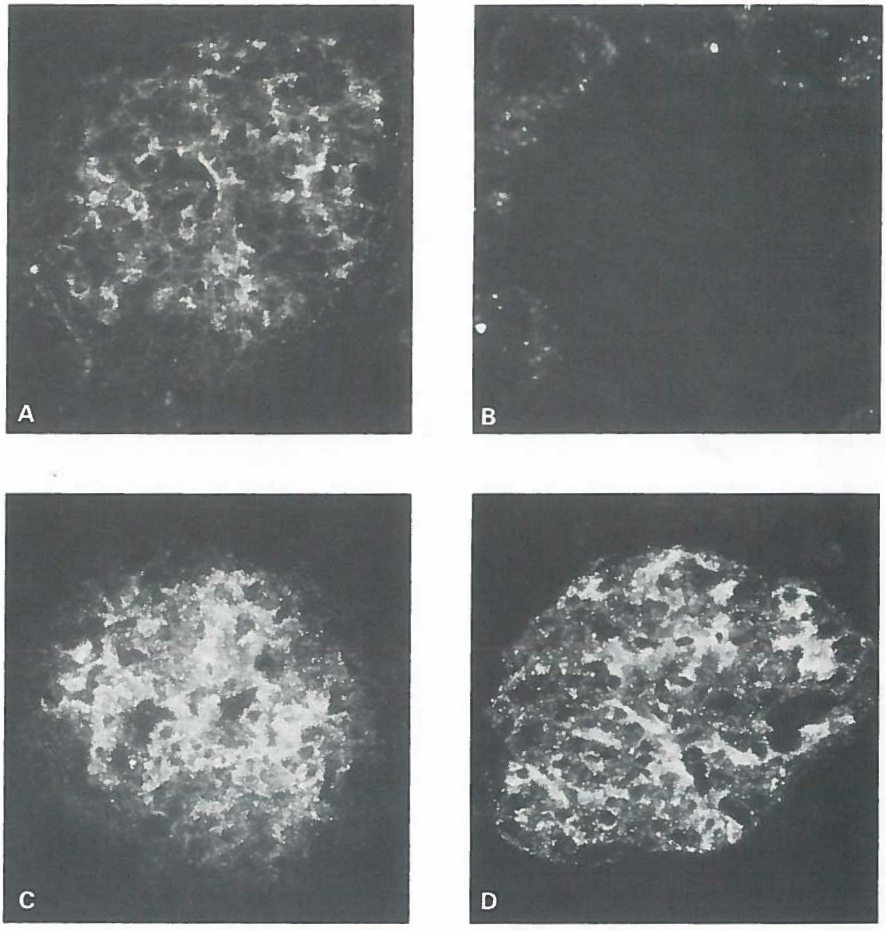


Figure 1
In vitro incubation of cryostat sections of normal rat kidney with 50 µg/ml of ER 14 (A), His 11 (B), ER 4 (C), or ER 5 (D), and FITC-conjugated goat anti-mouse IgG (1/40). All ER antibodies show confluent mesangial staining as well as fine granular staining along the capillary walls (A,C, and D: x400). {In addition some granular staining along Bowman's capsule is seen using ER 14 (A)}. Incubation with HIS 11 and FITC-conjugated goat anti-mouse IgG did not stain glomerular structures (B). Some autofluorescence of lysozomes in tubular structures can be seen (B: x400).

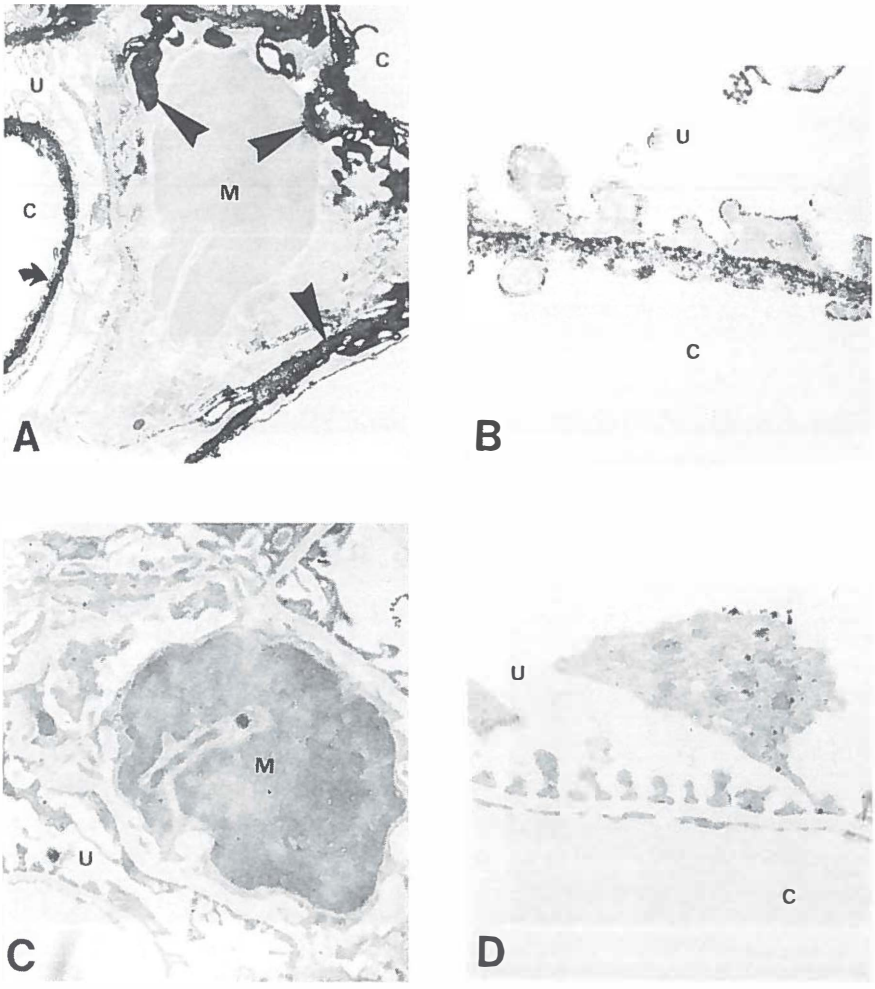


Figure 2
IEM of a normal rat kidney incubated with 0.5 ml containing 350 µg/ml ER 14 (A and B) or HIS 11 (C and D). Note the dark reaction product (arrowheads) present in the matrix of the mesangial cell (A: x7,140). Staining of the GBM and endothelial cell membranes is also observed (arrow). (B: x22,692) shows a peripheral part of the GBM. Reaction product is present throughout the GBM and along the membranes of the epithelial and endothelial cells. After incubation with His 11 no reaction product is detected (C: x9,044 and D: x18,091). (C: capillary lumen, M: mesangial cell, U: urinary space)

Immuno-electronmicroscopy (IEM). Binding of MoAbs to normal rat kidney at the ultrastructural level was studied using small pieces of 0.2% glutaraldehyde perfused kidney cortex. Cryostat sections of 16 μm were washed 5 times with PBS followed by incubation with 0.5 ml MoAb (350μ g/ml) for 3 h. Subsequently they were washed five times with PBS and incubated with 0.5 ml peroxidase conjugated rabbit antimouse IgG (1/20, Dakopatts, Denmark) for 3 h, followed by five washings in PBS. Then 3.3 diaminobenzidine (DAB) tetra-hydrochloride (0.1%) was added containing 0.01% H2O2; after 25 min incubation with DAB, the sections were washed with PBS (5x). All incubation steps so far were performed at room temperature. Postfixation in 0.1M phosphate buffered OsO4 for 45 min was done at 4C. The sections were embedded in Epon and processed according to standard methods [9]. Identical procedures were used for the detection of mouse IgG in kidneys which had been perfused with MoAbs.

RESULTS

Immunofluorescence microscopy.

After incubation of cryostat sections of normal kidneys with anti-Thy1.1 (ER 14) and FITC-GAM, mesangial staining, as well as a fine granular staining along the capillary walls, can be seen (Fig. 1A). In contrast, incubation with Moab directed to B cells (HIS 11) did not result in staining of glomerular structures (Fig. 1B). Incubation with ER 4 (Fig. 1C) and ER 5 (Fig. 1D) showed staining patterns similar to those seen using ER 14. Identical staining patterns (not shown here) occurred after perfusion into the rat kidney ex vivo of the Moabs used in this study- i.e. positive staining after perfusion of ER 4,5 and 14 and no detectable staining after perfusion of His 11.

Immunoelectron microscopy.

Incubation of normal rat kidney tissue with anti-Thy 1.1 (ER 14) showed reaction product in the mesangial matrix and along mesangial cell membranes, in particular in areas where the mesangial cell borders the endothelial cell membrane (Fig. 2A).

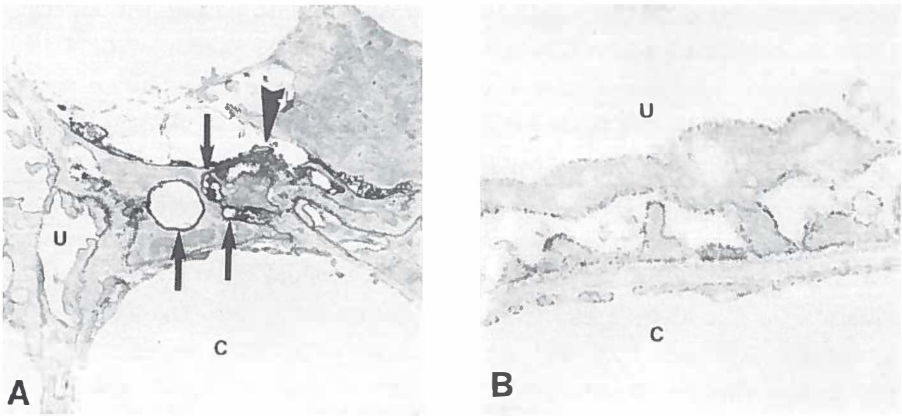


Figure 3
IEM of a rat kidney perfused with ER 14 (10 ml;100 µg/ml). Staining of mesangial channels (arrows) and matrix (arrowhead) (A: x7,526), as well as of the glomerular capillary wall (B: x25,480) can be observed. In the GBM some accumulation of reaction product can be seen in the lamina rara externa and lamina rara interna, whereas plasma membranes of endothelial and epithelial cells also stain positive. (C: capillary lumen, U: urinary space)

Reaction product can also be observed through the glomerular filtration barrier, including GBM and plasma membranes of endothelial and epithelial cells (Fig. 2B). After incubation with anti-B cell MoAb (His 11) no reaction product was observed in the mesangium (Fig. 2C) or along the capillary walls (Fig. 2D).
Perfusion of ER 14 into the kidney resulted in localization of reaction product in the mesangium similar to that observed after in vitro incubation (Fig. 3A). Reaction product was also detected in the capillary wall--i.e. through the GBM in particular in the lamina rara externa (LRE) and along the membranes of epithelial and endothelial cells (Fig. 3B). No reaction product could be detected after perfusion of anti-B cell MoAb (His 11) (results not shown).
Following perfusion with either ER 4 (Fig. 4A,B) or ER 5 (Fig. 5A,B) into the rat kidney ex vivo, reaction product along the mesangial matrix (Fig. 4A,5A), as well as throughout the GBM (Fig. 4B,5B), could be detected. After perfusion of ER 4, equal distribution of reaction product throughout the GBM can be detected (Fig. 4B), while after

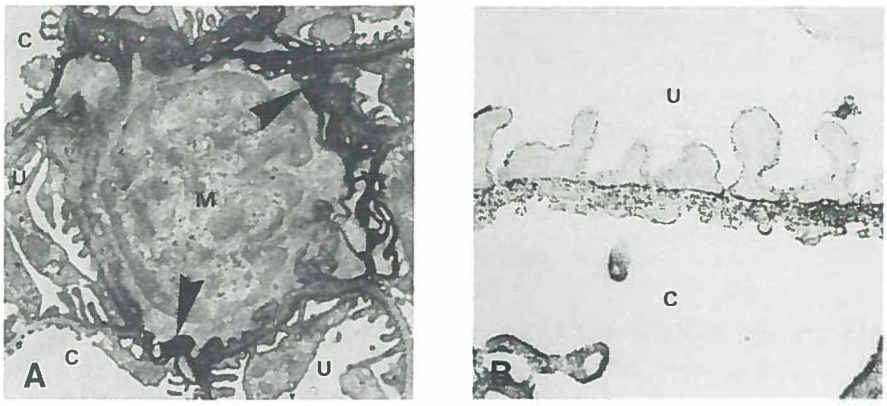


Figure 4
IEM of rat kidney after perfusion with ER 4. Prominent staining of the matrix of the mesangial cell (arrow-heads) (A: x7,705) is observed. Perfusion of ER 4 results in even distribution of reaction product in the GBM and staining of cell membranes of epithelial and endothelial cells (B: x21,112). (C: capillary lumen, M: mesangial cell, U: urinary space)

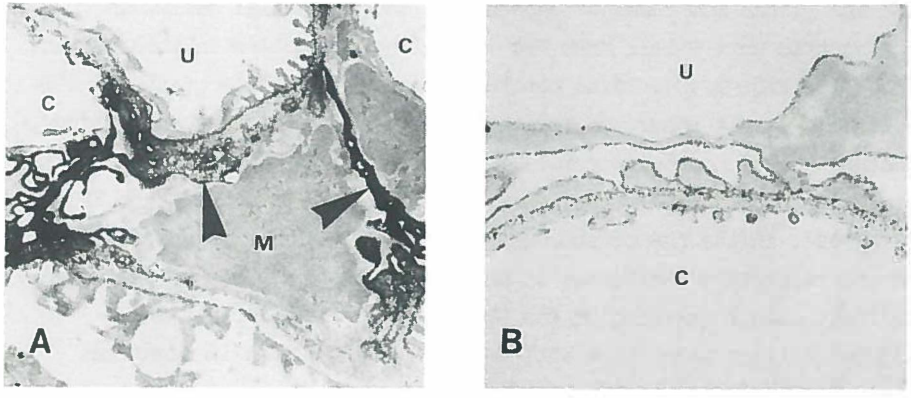


Figure 5
IEM of rat kidney perfused with ER 5. Staining of the matrix of the mesangial cell (A: x7,936) is observed (arrowheads). Epithelial and endothelial cell membranes are also stained, and reaction product can be seen in the GBM, in particular accumulated in the lamina rara externa (B: x 21,840). (C: capillary lumen, M: mesangial cell, U: urinary space)

perfusion of ER 5 reaction product can be seen in particular at the LRE of the GBM (Fig. 5B). Both antibodies also stained membranes of endothelial and epithelial cells. Identical staining patterns were seen after in vitro incubation of rat sections with ER 4 or ER 5 (results not shown).

DISCUSSION.

In the present study monoclonal anti-Thy 1.1 IgG 1 (ER 14) was compared with an anti-B cell MoAb of identical IgG 1 subclass (His 11) with respect to its binding properties to the rat kidney in vitro and after perfusion ex vivo. In addition, two more anti-T-cell MoAbs of different IgG subclass (ER 4 and ER 5) were studied for their binding properties in the rat kidney, as well as MoAbs of the IgG 2a subclass directed to rat leucaemic cells (pre-B cell leukemia [143]), kindly provided by Prof. Dr.P. Nieuwenhuis, Groningen. The latter anti-B MoAbs did not show staining in vitro or after perfusion ex vivo using IF (data not shown).

The results show binding of anti-T-cell MoAbs to mesangium as well as GBM and plasma membranes of endothelial and epithelial cells, in contrast to anti-B-cell MoAb His 11, which is negative in this respect (Fig. 1B; Fig. 2C,D). It is concluded that the subclass of the anti-T-cell MoAb is not important for the observed binding to the rat kidney. Whether the pronounced accumulation of reaction product after perfusion of ER 14 (Fig. 3B) and ER 5 (Fig. 5B), in contrast to ER 4 (Fig. 4B), in the LRE of the GBM reflects differences in specificity of the respective antibodies is uncertain. This possibility seems unlikely, since staining of the GBM after in vitro incubation of kidney sections with these anti-T-cell MoAbs resulted in identical staining patterns throughout the GBM (Fig. 2B).

The anti-T-cell MoAbs used in this study are directed to the Thy 1.1 antigens, since absorption with purified Thy 1.1 abolishes their anti-T-cell activity [144]. The tissue distribution of the antigen recognized by ER 14 is indeed identical to the distribution of MoAb OX 7 against purified rat Thy 1.1 [116]. However, since the Thy 1.1 molecule is characterized by a heterogenous glycosylation pattern [37], the three anti-T-cell MoAbs used may recognize different

epitopes of this molecule. This is probably also reflected by the fact that the anti-T-cell MoAbs do not stain identical subsets of lymphoid cells (Table 1).

Whether differences in epitope recognition by these anti-T-cell antibodies are also reflected in different distribution of reaction product in the GBM, as stated above (Figs. 3B, 4B and 5B)) cannot be deduced from the present data; moreover, it cannot be excluded completely, using these peroxidase techniques, that some diffusion of oxidized DAB plays a role.

Whatever the exact localization of the glomerular epitopes recognized by the anti-T-cell antibodies may be, from the IF data, as well as from the IEM results, it is clear that binding of anti-T-cell MoAb occurs in situ in mesangium, as well as within the GBM after contact with these MoAbs.

Since other glomerular constituents--i.e., type IV collagen, laminin or entactin--are also present in the tubular basement membrane [2, 27, 80, 98] which does not stain with the anti-T-cell MoAbs used, it is unlikely that our anti-T-cell MoAbs crossreact with these molecules. Similarly, crossreaction with actin [56, 118, 149] or fibronectin [51] seems unlikely, whereas these anti-T-cell MoAbs were not able to react with purified heparan sulphate using an in vitro assay (unpublished data).

In summary, the results of this study show that the GBM of the rat kidney contains T-cell-like epitopes, and they confirm the presence of Thy 1.1 antigen in the mesangium of the rat kidney, as demonstrated by other authors [81, 97, 123, 136]. This implicates that immune complex formation in situ after in vivo injection with polyclonal ATS, as shown previously [9], may be due to these T-cell epitopes rather than to contaminating anti-connective-tissue antibodies, a mechanism generally assumed to explain the nephritogenicity in some batches of ATS prepared for clinical use [120, 161]. The potential significance of T-cell epitopes in the rat kidney is illustrated by massive proteinuria observed after a single injection of ER 4 (to be reported separately).

ACUTE GLOMERULONEPHRITIS AFTER INTRAVENOUS INJECTION OF MONOCLONAL ANTI-THYMOCYTE ANTIBODIES IN THE RAT

W.M. Bagchus, Ph.J. Hoedemaeker[1], J. Rozing[2] and W.W. Bakker

Department of Pathology, University of Groningen, Groningen.
(1) Department of Pathology, University of Leiden, Leiden,
(2) Department of Internal Medicine, Erasmus University, Rotterdam.

SUMMARY

Female Wistar rats were injected with (mouse) monoclonal antibodies (Moabs) of different IgG subclasses directed to rat thymocytes or rat tumor cells. Following intravenous injection of anti-thymocyte Moabs, glomerular binding of mouse IgG was observed during the first 4 days along the GBM and in the mesangium. No staining for mouse IgG was detected in anti-tumor MoAb injected rats. Animals injected with IgG 2a anti-thymocyte Moab developed glomerulonephritis and a massive proteinuria in contrast to rats injected with IgG 1 Moab which is non-complement fixing. The glomerulonephritis lesion consisted of microaneurysms and focal and segmental proliferation. Deposits of complement and fibrin could be detected exclusively in rats injected with IgG 2A anti-thymocyte MoAb during the whole observation period of 14 days. This is the first demonstration of overt glomerulonephritis lesions on the injection of monoclonal antibodies.

INTRODUCTION

Anti-human thymocyte globulin used in the clinical situation as an immuno-suppressive agent may contain anti-kidney antibodies [34]. These anti-kidney antibodies may be induced by stromal contamination of the inoculum used for immunization [120].

Recent investigations in our laboratory have shown that polyclonal rabbit anti-rat thymocyte antibodies also contain anti-kidney antibodies that bind to rat kidney in vitro or in vivo following intravenous injection as well as after perfusion in the normal rat kidney ex vivo [9]. In addition, we have shown that MoAbs directed to rat thymocytes, in contrast to anti-rat B cell MoAbs, are able to recognize glomerular structures of the rat kidney in vitro and after perfusion ex vivo [11]. From these data it can be concluded that epitopes are shared between thymocytes and rat kidney structures. For this reason it might be expected that these anti-thymocyte Moabs could have nephritogenic properties in vivo. Therefore, we studied the effect of injecting rats with two different (mouse) monoclonal anti-thymocyte antibodies (of IgG 1 and IgG 2a subclass respectively) or with (mouse) monoclonal anti-tumor antibody (IgG 2a subclass). The results show that intravenous injection of anti-thymocyte Moabs studied with subclass IgG 2a leads to massive proteinuria and glomerulonephritis, while injection of the anti-thymocyte Moab of IgG 1 subclass or anti-tumor Moab did not. Of the last two Moabs, the anti-thymocyte Moab, however, did show a binding to glomerular structures in a similar pattern as the disease inducing anti-thymocyte MoAb of the IgG 2a subclass.

MATERIAL AND METHODS

Experimental design. Three groups of female Wistar rats 6 wk of age were injected i.v. with 0.5 ml saline containing 400 μg protein of ascites derived ammonium sulphate precipitated MoAb. Rats in group A (n=12) received MoAb ER4, rats in group B (n=6) received MoAb ER 14 and those in group C received control MoAb His 32 (n=4). The characteristics of these MoAbs are summarized in table 1.

Proteinuria. The animals were housed in metabolic cages, and protein excretion was measured daily, using the biuret method.

Immunofluorescence- and light microscopy. Two and 24 h and 4 and 14 days after i.v. injection, kidneys of experimental and control rats

Table 1
Characteristics of antibodies used for intravenous injection in Wistar rats.

1 Code	Subclass	2 Antigenic determinant	Complement binding capacity	3 Recognition by FACS analysis
ER 4	IgG 2A	25 kD	yes	98% thymocytes 1% spleen cells 5% PBL 20% bone marrow cells
HIS 32	IgG 2a	nd	yes	rat pre-B-cell leukemia
ER 14	IgG 1	25 kD	no	98% thymocytes 3% spleen cells 0% PBL 31% bone marrow cells

PBL = peripheral blood lymphocytes, nd = not determined

1) The ER coded antibodies were prepared by Dr J. Rozing, Erasmus University Rotterdam. MoAb HIS 32 was a gift from Prof. Dr.P.Nieuwenhuis, Department of Histology, Groningen.
2) Molecular weight of antigenic determinants, as detected by immuno-precipitation is indicated in kilodaltons (kD) [144].
3) Percentage of lymphoid cells recognized by the ER antibodies as determined by FACS-analysis with fluorescein isothiocyanate (FITC)-conjugated IgG [144]. The FACS data were confirmed by immuno- histology. The tumor specificity of HIS 32 was determined by immuno-histology (peroxidase method) [143].

were processed for light microscopy and immunofluorescence microscopy, according to standard procedures. Kidneys were examined for the presence of mouse IgG, rat complement and rat fibrin in the glomeruli using FITC-conjugated goat anti-mouse IgG (1/40), FITC-conjugated goat anti rat-C3 (1/20) or FITC-conjugated goat anti-rat fibrin (1/40), respectively, (Nordic, Tilburg, The Netherlands). Sections for light microscopy were stained with haematoxylin and eosin, Periodic acid Schiff or methamine silver.

Immunofluorescence microscopy

All animals in group A showed mouse IgG in their mesangium and to a lesser extent fine granular staining along the capillary walls 2 h after i.v. injection of Moab ER 4 (Fig. 1).

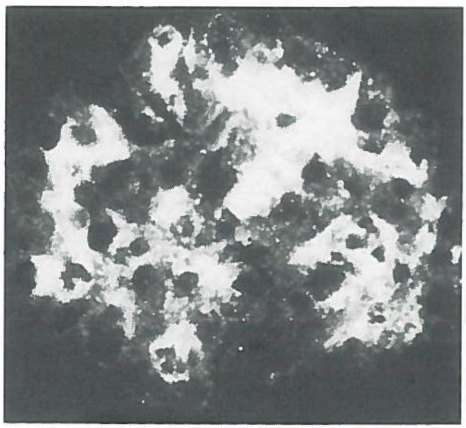


Fig. 1
Glomerulus of a rat kidney 2 h after injection i.v. with 400 µg Moab ER4, stained with FITC-conjugated goat anti-mouse IgG. Prominent staining of the mesangial area and to a lesser extent fine granular staining along the glomerular capillary wall is observed (x 560).

After 24 h and 4 days the staining intensity was clearly diminished and at day 14 mouse IgG could no longer be detected. Kidneys of rats injected with Moab ER 14 showed identical glomerular distribution of mouse IgG, which no longer could be detected after day 4. None of the rats injected with Moab His 32 had mouse IgG in their glomeruli. Two h after i.v. injection of Moab ER 4 fibrin and C3 was detected in all glomeruli and remained present during the whole observation period. Kidneys from rats from group B and group C stained negative for fibrin or complement.

Light microscopy

All animals injected with Moab ER4 demonstrated glomerulonephritis upon light microscopy. Two h following the injection the glomeruli showed nuclear cell debris resembling apoptotic bodies in both the mesangial regions as well as in capillary loops (fig 2).

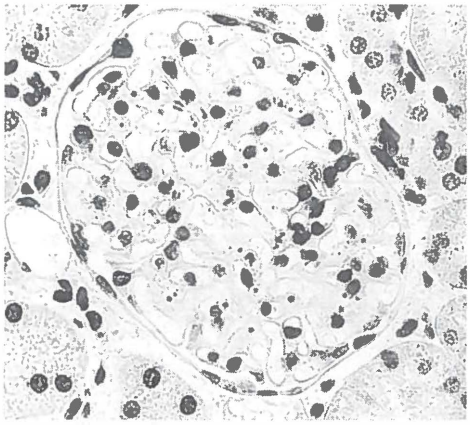


Fig. 2
Glomerulus of the same rat of fig 1. Several small apoptotic bodies can be seen in the mesangium as well as in glomerular capillary loops. (Haematoxylin and eosin, x 560.)

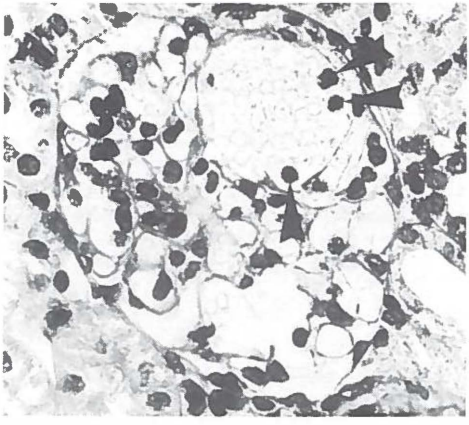


Fig. 3
Glomerulus of rat kidney 4 days after ER 4 injection showing microaneurysms of the capillaries. In the `ballooning' microaneurysm erythrocytes as well as some inflammatory cells can be seen (arrowheads). (Periodic acid Schiff, x 560)

A few glomeruli showed polymorph nuclear cells (PMN's). No tubulo-interstitial abnormalities could be seen in kidneys of these animals. After 24 h nuclear cell debris was no longer detectable and increased influx of inflammatory cells (including mononuclear cells and PMN's) could be seen in most of the glomeruli of these rats. Four days after iv injection of ER4 the majority of glomeruli showed increased cellularity as well as microaneurysms of various sizes (`ballooning') (Fig. 3). At day 14, in addition to glomerular changes, interstitial lesions were observed. Some mononuclear cell infiltrates were present in the interstitium and also atrophic tubuli could be observed (Fig 4).

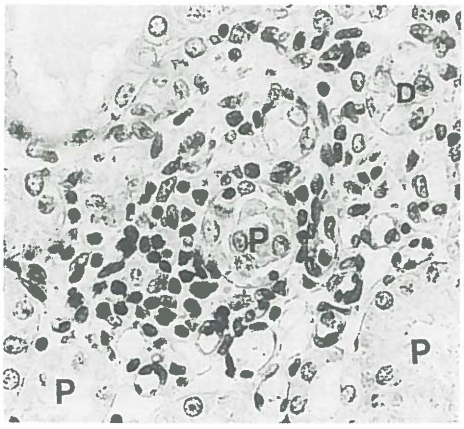


Fig. 4
Kidney section of a rat 14 days after ER4 injection, showing interstitial mononuclear inflammatory cells affecting a proximal tubule.
P = proximal tubule, d= distal tubule (Haematoxylin and eosin, x 560)

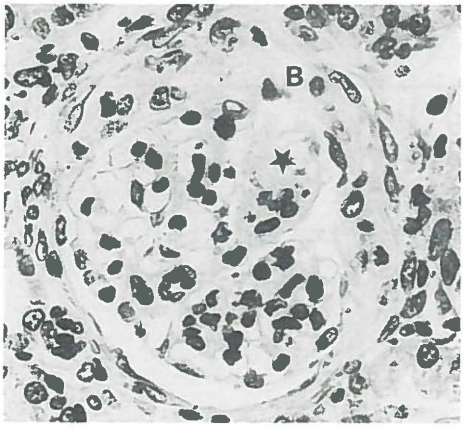


Fig. 5
Glomerulus of same rat kidney as in Fig. 4. Segmental proliferation in mesangial areas (asterisk) and proliferation of Bowman's capsule (B) is observed. (Periodic acid Schiff , x 560)

At this time the glomeruli showed an increase of cells in mesangial areas and thickening of Bowman's capsule with crescent-like formations (Fig 5 and 6). None of the animals injected with MoAb HIS 32 or MoAb ER 14 showed histological abnormalities in their kidneys.

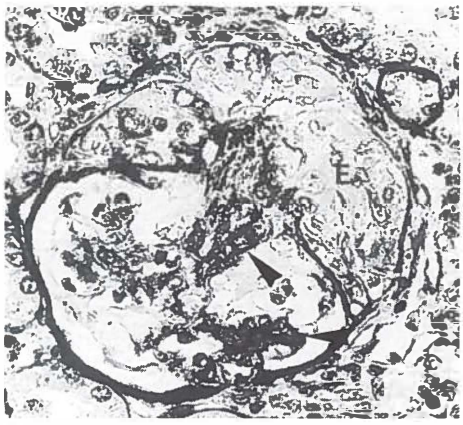


Fig. 6
Glomerulus of a rat kidney from ER4 injected rat after 14 days, showing increase of mesangial matrix (arrowheads) and extra-capillary proliferation giving rise to a crescent-like formation. E = extracapillary proliferation. (Methamine silver, x 560)

Proteinuria

In table 2 the mean protein excretion of rats of group A is depicted at several intervals during the observation period. All rats in group A showed proteinuria, in contrast to the rats in groups B and C where protein excretion did not exceed 5 mg/ 24 h (data not shown). Though in general mean protein excretion gradually decreased during the observation period after day 4, two individual rats of group A still had protein excretion exceeding 90 mg/24 h at day 14.

Table 2
Mean protein excretion of rats injected with ER 4 (group A)

Days after i.v. injection	*Urinary protein (mg/24 h ± 1 x SD)*
1	*36 ± 15*
4	*320 ± 34*
7	*181 ± 36*
10	*103 ± 36*
14	*47 ± 33*

SD = standard deviation

DISCUSSION

The present study demonstrates that a single injection of a relatively small amount [96, 139] of anti-thymocyte MoAb of the IgG 2a subclass can induce an acute glomerulonephritis in Wistar rats with considerable proteinuria and histopathological alterations. Anti-thymocyte MoAb of a non-complement binding subclass or control Moab do not show this ability.

In previous experiments we demonstrated in situ binding of Moab ER 4 to the GBM and mesangium, of the normal rat kidney after perfusion ex vivo [11]. This finding, and the observation in this study of a direct glomerular binding of mouse IgG after injection of Moab ER 4, support the idea that the in vivo activity of Moab ER4 also is initiated by in situ binding of this antibody to glomerular epitopes.

Although contribution of immune complexes (formed in the circulation after ER 4 treatment) to the pathogenesis of the present disease cannot be excluded completely, this possibility is unlikely since no mouse IgG could be detected in the liver of these animals (results not shown). Since both anti-thymocyte MoAbs tested in this study bind in a similar pattern in the rat glomeruli after i.v. injection and only Moab ER4 is able to activate complement, it is likely that the inflammatory reaction and proteinuria induced by ER4 is a consequence of complement activation in vivo.

This glomerulonephritis shows similarities with other experimental forms of glomerulonephritis, caused by binding of antibody either to the GBM [76] or to an antigen planted in the GBM [163] in the rat, or the Arthus-type nephritis in the rabbit [72, 155].

To our knowledge this is the first demonstration that complement binding monoclonal antibodies are able to induce an acute glomerulonephritis. This induction probably is caused by shared epitopes between the GBM of the rat kidney and rat thymocytes.

GLOMERULONEPHRITIS INDUCED BY MONOCLONAL ANTI-THY 1.1 ANTIBODIES. A sequential histological and ultrastructural study in the rat

(1) (2) (3) 1
W.M. Bagchus, Ph.J. Hoedemaeker, J. Rozing and W.W. Bakker

(1) Department of Pathology, University of Groningen, Groningen.
(2) Department of Pathology, University of Leiden, Leiden.
(3) Institute for Experimental Gerontology,
Organization for Health Research TNO, Rijswijk.

SUMMARY

The present report describes the natural history of an experimental acute glomerulonephritis with massive proteinuria induced by a single intravenous injection of a (mouse) monoclonal anti-rat Thy 1.1 antibody into the rat. The disease is characterized by direct although transient binding of this monoclonal antibody to glomerular basement membrane and mesangium after injection as demonstrated by immunofluorescence microscopy. Immediate activation of complement occurs as shown by glomerular deposition of C3 and C9. Concomitant activation of the coagulation cascade is reflected by the presence of fibrinogen depositions in the affected glomeruli. One hour after injection mesangial alterations are prominent including condensation of mesangial cell chromatin, and lysis of mesangial cells as shown by light- and electron- microscopy, leading to the formation of aneurysms in the capillary tuft. From day 4 on mesangial cell proliferation can be observed, accompanied by glomerular crescent formation at day 14, which decreases gradually 3 weeks after antibody administration, whereas mesangial hypercellularity can be observed up to week 10 after i.v. injection of the antibody. The disease is clinically characterized by a massive transient proteinuria starting immediately after antibody injection, reaching mean values of 300 mg/24 h at day 2-4, gradually decreasing till normal levels after 3 weeks. It is concluded that in this unique model of glomerulonephritis induced by a monoclonal antibody, recognition of Thy 1.1 epitopes as well as

activation of complement including the C5-C9 membrane attack complex may play a major role in the pathogenesis of this experimental disease.

INTRODUCTION

It has been demonstrated by several authors [97, 123, 136] as well as in our laboratory that T-cell epitopes are present in glomerular structures of the rat kidney [9]. Using both in vitro incubation of kidney tissue with monoclonal (Moab) anti-Thy 1.1 IgG or perfusion of these antibodies into the normal rat kidney ex vivo, evidence was obtained that epitopes of the Thy 1.1 antigen are present predominantly in the mesangial area [12, 136] and to a lesser extent along the glomerular basement membrane (GBM) of the rat kidney [12]. Although in vivo administration of either polyclonal [9] or monoclonal [13] anti-thymocyte antibodies resulted in glomerular binding as demonstrated by immunofluorescence, it has been shown that only one of the anti-Thy 1.1 Moabs used (ER 4) was able to induce acute glomerulonephritis and proteinuria. To further characterize this glomerulonephritis induced by a single injection of a relatively low amount of anti-T Moab we now report on the natural history of this model and on the glomerular alterations observed at the ultrastructural level.

MATERIAL AND METHODS

Antibody. The main characteristics of the ER4 antibody used for intravenous (i.v.) administration and prepared by Dr.J. Rozing are summarized in table 1.

Experimental design. Female Wistar rats 8 weeks of age were injected intravenously (i.v.) with 0.5 ml saline containing 400 μg of ascites derived ammonium sulphate precipitated Moab ER4. Kidneys of rats were studied by sacrificing groups of three rats at 1, 2 and 24 h and 4,6 and 14 days after i.v. injection of Moab. After this period until the

end of the observation period at week 10, two rats were sacrificed every week and their kidneys were studied.

Proteinuria. The animals were housed in metabolic cages and protein excretion was measured daily for the first 14 days, using the biuret method. The next 8 weeks protein excretion was measured weekly.

Table 1. The characteristics of ER4 antibody used for intravenous injection in Wistar rats.

Code	Subclass	Antigenic determinant	Molecular weight	Complement binding capacity	Percentage lymphoid cells recognized [1]
ER 4	IgG 2A	Thy 1.1	25 kD	+	97% thymocytes 1% spleen cells 1-3% PBL 20% bone marrow cells

1) Percentage of lymphoid cells from adult rats recognized by the ER4 antibody as determined by FACS-analysis with fluorescein isothiocyanate (FITC)-conjugated IgG [173]. The FACS data were confirmed by immuno-histology [103].
PBL= peripheral blood lymphocytes.

Immunofluorescence-, light- and electron-microscopy. Kidneys were processed for immuno-fluorescence microscopy (IF), light microscopy and electron microscopy according to standard procedures. Fluorescein isothiocyanate (FITC)-conjugated antisera were purchased from Nordic, Tilburg, The Netherlands. Kidney sections were examined for the presence of mouse IgG, rat complement and rat fibrin in the glomeruli using FITC-conjugated goat anti-mouse IgG (dilution 1/40), FITC-conjugated goat anti rat-C3 (1/20) and FITC-conjugated goat anti-rat fibrinogen (1/20). The presence of rat IgG in kidney sections was studied using FITC-conjugated rabbit-anti-rat IgG (1/40). To avoid crossreaction with mouse IgG, this conjugated antiserum was supplemented with 1% normal mouse serum before use.

C9 Deposits in rat kidney sections were examined using rabbit antiserum (1/5) against aggregated human C9 with FITC-conjugated goat-anti-rabbit IgG (1/60) as a second step. This rabbit antiserum, a kind gift from Dr. M.R. Daha, Leiden, The Netherlands, is known to crossreact with rat C9 (Dr.Daha, personal communication).
Sections for light microscopy were stained with haematoxylin and eosin (HE), periodic acid Schiff or methanamine silver. For electron microscopy pieces of kidney cortex (1 mm3) were fixed in 2% glutaraldehyde in phosphate buffered saline pH 7.38 processed according to standard methods and stained with 5% uranyl acetate and 3% lead citrate.

RESULTS

Proteinuria

In Fig. 1 the daily mean protein excretion of ER4 injected rats is depicted for the first 14 days (left) and the weekly mean protein excretion for the next 8 weeks (right). All rats showed proteinuria during the first 14 days. The mean weekly protein excretion observed at week 3 and during the remaining observation period falls within the range observed in normal female age-matched Wistar rats.

Immuno-fluorescence microscopy

All animals showed mouse IgG in their mesangium and to a lesser extent fine granular staining along the capillary walls one and two h after i.v. injection of Moab ER 4. Staining intensity decreased gradually and after 6 days mouse IgG could no longer be detected. Directly after i.v. injection of the antibody fibrinogen and C3 was detected in all glomeruli. The amount of C3 increased during the first two h, then slowly decreased leaving only a faint staining on day 6. At day 14 prominent staining for C3 was observed, especially in glomerular crescents followed by a gradual decrease. From week 5 until week 10 only a few glomeruli showed traces of C3.
Fibrinogen staining increased gradually from 60 minutes up to day 4, and was prominent from day 4 to week 3. After the fifth week the percentage of positive glomeruli decreased gradually, although ten weeks after i.v. injection of ER4 fibrinogen was still present in a few glomeruli in each kidney section studied.

One and two h after i.v. injection of the antibody, extensive staining for C9 was noted in the same pattern as observed for mouse IgG (Fig. 2) :i.e. prominent mesangial staining and faint staining along the GBM.

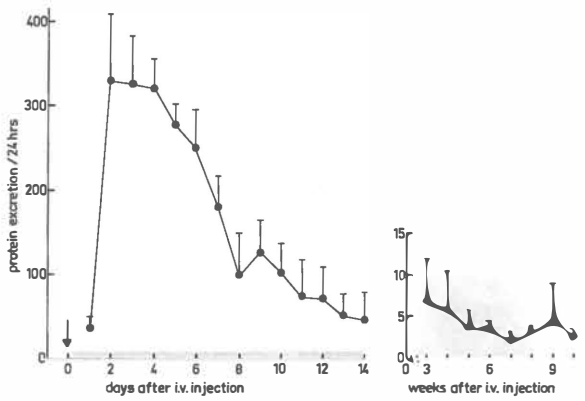


Fig. 1. Mean 24 hour protein excretion ± 1 x standard deviation is shown. Protein excretion was measured daily for the first 14 days, (left) and weekly after that period till the tenth week (right). Note the different scales used. Shaded areas show the range of protein excretion observed in normal female age-matched Wistar rats. Arrow indicates i.v. injection of 400 µg ER4 at day 0.

From this moment on the staining gradually decreased, reaching background levels at day 4. However at day 14 some glomeruli showed again foci of positive staining for C9, decreasing rapidly thereafter. From week 3 to 5 only a few glomeruli showed traces of C9.
Because no increased staining for rat IgG was present in kidney sections taken for the first 4 days after ER 4 injection, it was concluded that the addition of 1% normal mouse serum abolished the crossreaction of FITC-conjugated rabbit anti-rat IgG with mouse IgG. At day 6 a few glomeruli showed traces of rat IgG. From day 14 rat IgG was present in those glomeruli showing extensive fibrinogen staining.

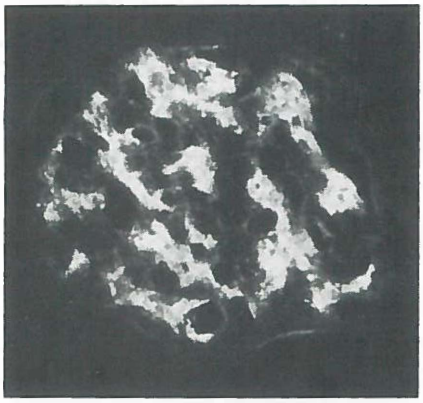


Fig. 2. Glomerulus of a rat kidney 2 h after i.v. injection of ER4 stained with rabbit anti-C9 and FITC-conjugated GAR. Positive staining is observed in the mesangium and faintly along the GBM. (x 400)

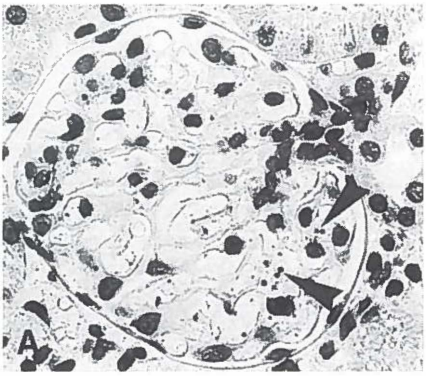
Light microscopy and electron microscopy

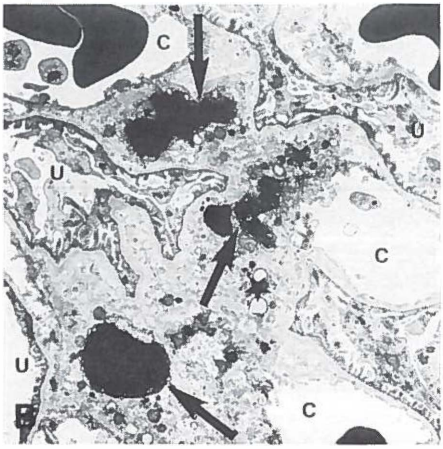
According to the observed morphological aspects of the mesangial cell, we arbitrarily divided the light and ultrastructural findings in stages, which succeeded each other in a fairly orderly fashion.

Stage I

At 1 and 2 h after i.v. injection, condensation of nuclear chromatin of the mesangial cell was observed. At the histological level, this condensation of chromatin resembles apoptotic bodies (Fig. 3A).

No additional glomerular or tubulo-interstitial abnormalities could be seen in kidneys of these animals. At the ultrastructural level, one hour after i.v. injection, in all mesangial areas condensation of chromatin and also condensation and fragmentation of cytoplasmic organelles of mesangial cells was observed (Fig. 3B).
Two h after i.v. injection in addition to apoptotic bodies there are areas with necrotic resolving mesangial cells (Fig. 3C). A few glomeruli showed erythrocytes in the urinary space, indicating increased glomerular capillary wall permeability (Fig. 3C).





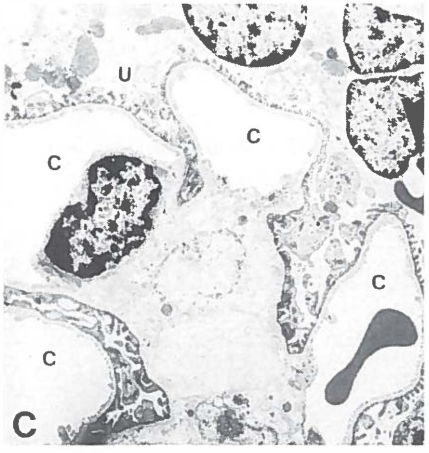


Fig. 3. Glomerulus of a rat kidney following injection of ER4. Stage I.
A) Two h after i.v. injection several small dark round dots (apoptotic bodies) are observed mainly in mesangial cells (arrowheads). (x400) HE-staining.
B) EM photograph of a glomerulus of a rat after 60 min, showing condensation of chromatin material (arrows), and condensed mesangial cytoplasm (asterisk) (x3,218).
C) Electron micrograph 60 min after i.v. injection. A resolving mesangial cell is observed, covered by a normal endothelial cell. An erythrocyte is observed in the urinary space (arrow head), (x3,218).
C= capillary lumen, En=endothelial cell, U=urinary space.

Stage II

Twenty-four h after injection of ER4 partial disappearance of mesangial cells is observed. Fig. 4A shows an empty mesangial area as well as remnants of mesangial cells or matrix. At the light microscopic level, increased influx of inflammatory cells (mononuclear cells and polymorph nuclear cells) could be seen in most of the glomeruli of these rats (Fig. 4B). Apoptotic cell bodies are no longer observed.

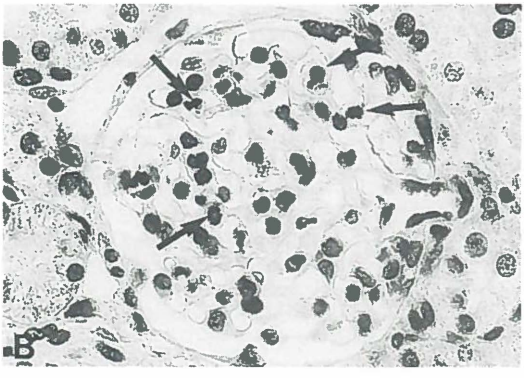


Fig. 4. Glomerulus of a rat kidney following injection of ER4. Stage II.

A) Electron micrograph 24 h after i.v. injection; the glomerulus contains areas with remnants of mesangial matrix (arrowhead) next to completely dissolved mesangial cells (asterisk) (x 4500). C= capillary lumen, En=endothelial cell, Ep=epithelial cell, U= urinary space.

B) Light microscopy of a glomerulus from a rat kidney 24 h after ER4 administration, showing the presence of polymorph-nuclear cells.(arrows) (x 400), HE stain.

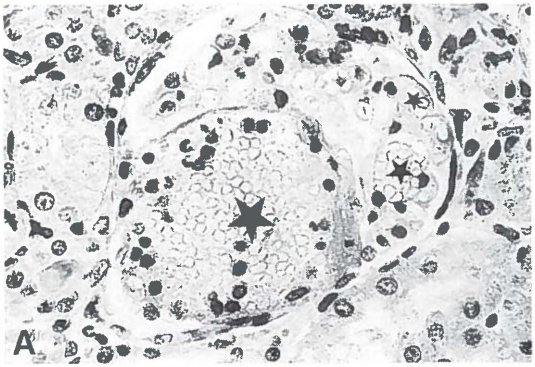
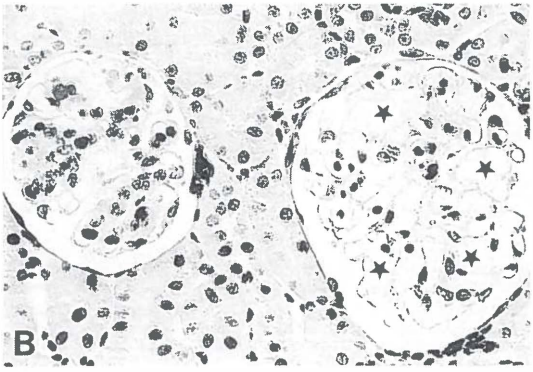
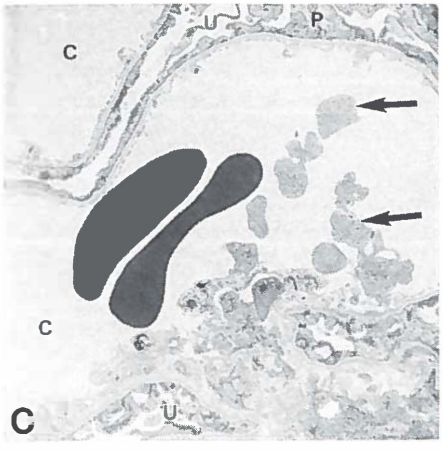


Fig. 5. Glomerulus of a rat kidney following injection of ER4.
Stage III.

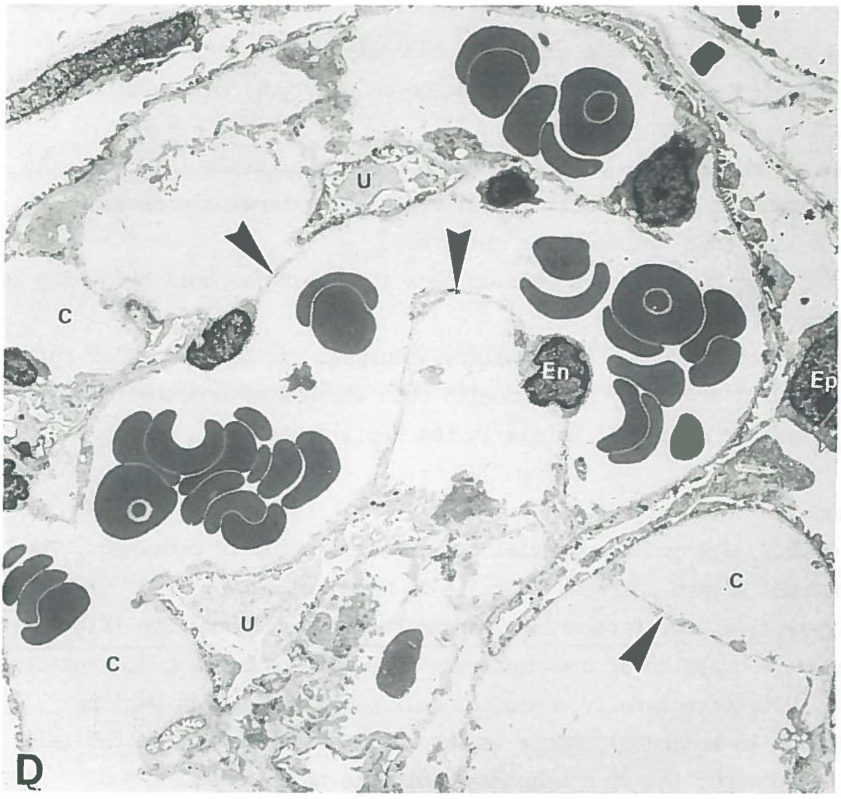
A) Glomerulus 4 days after i.v. injection. Small (small asterisks) as well as relatively large capillary aneurysms (large asterisk), filled with red blood cells, inflammatory cells and some fibrin, can be observed. (x 400), HE-stain



B) Light microscopy of rat kidney 6 days after i.v. injection of ER4. The glomerulus on the right shows capillary aneurysms (asterisk), in the left glomerulus mesangial proliferation can be seen. (x 300)



C) 4 Days after ER 4 injection electronmicroscopy of a glomerulus showing two capillary loops with prominent fusion of epithelial foot processes, ; platelets (arrows) near the mesangial area can be seen. (x 4,838)
C= capillary lumen, P=podocyte, U=urinary space.



5D) *Electron-microscopy of a part of the glomerulus at stage III showing aneurysms of capillary loops filled with erythrocytes. Thin strands of detached endothelial cell cytoplasm in the capillary lumen are prominent. (arrowheads) (x2300))*
C= capillary lumen, En=endothelial cell, Ep=epithelial cell, U=urinary space.

Stage III

At 4 and 6 days in most glomeruli mesangial cells have totally disappeared, leading to the formation of aneurysms of the capillary loops (Fig. 5A,B).

Other glomeruli show proliferation of mesangial cells (Fig. 5B). At this stage at the ultrastructural level, an extensive effacement of epithelial foot processes is observed.

In addition platelet aggregation near the mesangial cell (Fig. 5C) is present.

Fig. 5D demonstrates the capillary aneurysms of the glomerular tuft, filled with erythrocytes, and with thin strands of endothelial cytoplasm or mesangial matrix in the capillaries.

Stage IV

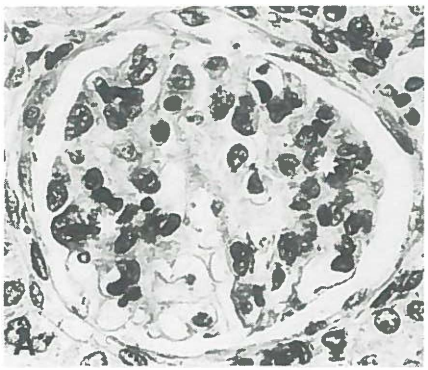
At week 2, 3,4 and 5 mesangial cell proliferation is prominent. The glomeruli showed increase of cells in mesangial areas (Fig. 6A) and extracapillary proliferation leading to crescent formation (Fig. 6B). Gradual regression of crescents was observed between the 3rd and 5th week. Ultrastructurally mesangial cell proliferation as well as increase in mesangial matrix is observed; effacement of epithelial foot processes is not a regular finding in this stage.

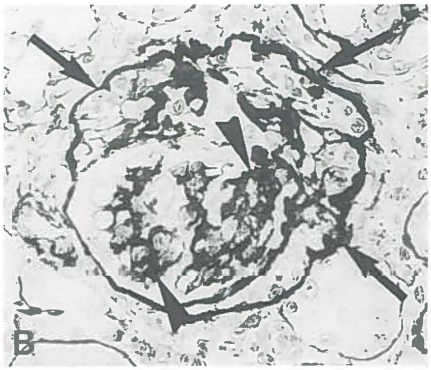
In addition, interstitial changes are observed which are characterized by mononuclear infiltrates in the interstitial tissue and atrophic tubuli (Fig. 6C).

These interstitial lesions were seen up till the 5th week.

Stage V

The only changes seen at this stage (week 6-10) are the persistence of the hypercellularity (Fig. 7A) and increased mesangial matrix, often containing collagenous fibrils, as observed at the ultrastructural level (Fig. 7B).





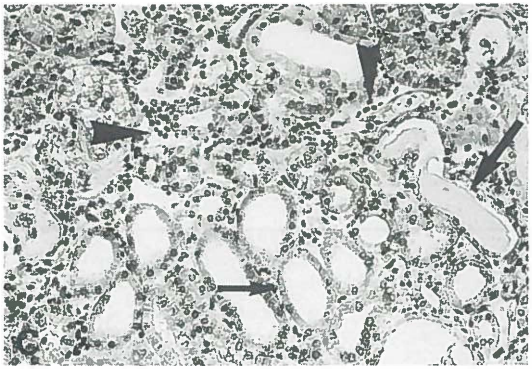


Fig. 6. Rat kidney section following injection of ER4. Stage IV.

A) Light microscopy of a glomerulus of a rat kidney, 14 days after i.v. injection, showing proliferation of cells in mesangial areas (asterisk) and thickening of Bowman's capsule (arrowhead). (x730) HE-staining

B) Methanamine silver staining of a rat glomerulus 14 days after injection of ER4. Crescent-like formation of basement membrane material is observed near Bowmans capsule (arrows). Dark staining is also observed in mesangial areas (arrowheads) indicating production of mesangial matrix. (x730) MS-stain.

C) Histological appearance of the cortex of a rat kidney 14 days after i.v. injection of ER 4. Atrophic tubuli (short arrow), tubuli filled with proteinacious material (long arrow) and normal tubuli are observed. Infiltrating mononuclear cells can also be seen.(arrowheads) (x100)

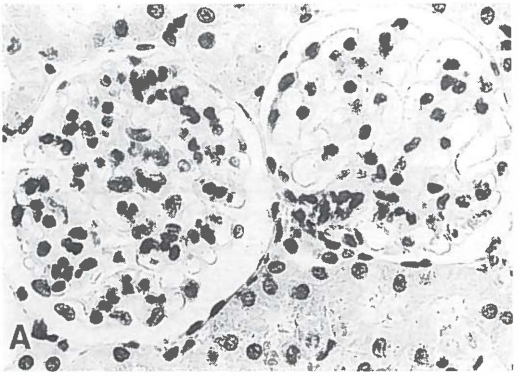


Fig. 7 Glomeruli of rat kidney following injection of ER4.
Stage V.

A) Two glomeruli of a rat kidney, ten weeks after injection. On the right a glomerulus with slightly increased mesangial matrix can be seen, on the left a glomerulus with mesangial hypercellularity can be observed. (x300)

B) Electron micrograph of part of a rat glomerulus showing mesangial cells with increased mesangial matrix, containing collagenous fibrils (arrows). (x 5,006) U=urinary space, M=mesangial cell

DISCUSSION

The present data confirm and extend the observation that a single injection of a monoclonal antibody (ER4) directed against an epitope on the Thy 1.1 antigen is able to induce acute glomerulonephritis in the rat [13]. This disease is characterized by massive though transient proteinuria, direct binding of mouse IgG and complement to mesangium and GBM and a series of subsequent glomerular alterations including mesangiolysis (Fig. 3C,4A,5D) and extracapillary proliferation (Fig. 6B) during the first 2 weeks, gradually converting in mesangial hypercellularity, during the next weeks (Fig. 5B,6A and 7A,B).

The nephrotoxic effect of this Moab is initiated by a direct binding of anti-Thy 1.1 antibodies to the rat mesangial cell membrane. The possibility that circulating immune complexes formed between Moab and shedded cell Thy 1.1 antigens [173] play a role is unlikely for three reasons: 1) in the rat, in contrast to the mouse, only a few PBL's carry the Thy 1.1 epitope; 2) no mouse IgG could be detected in the liver of these animals; 3) ex vivo perfusion experiments into the blood free rat kidney have shown that this Moab binds in situ to the rat mesangium and GBM [12].

The prominence of C9 (Fig. 2), as well as the presence of C3, in rat glomeruli after binding of ER4 is concomitant with the beginning destruction of mesangial cells in stage I. This indicates that activation of the membrane attack complex of complement [137] plays a prominent role in the lysis of the mesangial cell. This is in agreement with our previous study showing absence of mesangiolysis using non-complement binding anti- Thy 1.1 Moabs [13], and with recent publications showing that complement depletion by Cobra Venom Factor abolishes glomerulonephritis in rats induced by polyclonal anti-Thy 1.1 antibodies [179].

The mesangiolysis observed in all experimental animals results in the loss of 'anchoring points` of the GBM in the mesangium, leading to dilatation of capillary lumina, attaining aneurysmatic proportions, of different sizes, as observed in stage III. This is followed by segmental hypercellularity (Stage III, IV and V). These glomerular alterations bear a striking resemblance to the findings in the Habu Snake Venom model in the rabbit, i.e., mesangiolysis, aneurysms and

segmental hypercellularity [121, 122]. However, in the rat mesangiolysis after injection of Habu Snake Venom is not a common finding [31, 39, 49]. In contrast, both in the rabbit and in the rat mesangial cell proliferation is observed after a stage with capillary ballooning in the Habu Snake Venom model. Whether in our model segmental hypercellularity is influenced by the release of platelet factors [40, 41] or due to the loss of heparin-like molecules in GBM and mesangial matrix [49], remains to be established.

Glomerular hypercellularity persisted in our model for 10 weeks. No glomerular sclerosis was found during this period. These observations are in agreement with findings in the Habu Snake model [121]. Ultrastructural observations of glomeruli 7 to 10 weeks after i.v. injection showed collagenous fibers in the mesangial matrix. A similar finding was reported by Hsu [95] after cyclophosphamide induced mesangiolysis in the mouse.

In the present model a clear correlation between mesangiolysis and proteinuria exists, in contrast to other experimental models with mesangiolysis, in which this correlation remains obscure, i.e. Habu Snake Venom [121] or mesangiolysis induced by toxic agents [95]. Interestingly in the human situation mesangiolysis is also often associated with proteinuria [122]. Despite this correlation in the present model, the mechanism by which mesangiolysis would contribute to the increased glomerular permeability remains to be established.

One and 2 h after i.v. injection of ER 4, nuclear degradation of cells leading to the formation of apoptotic bodies are seen [152], not only in glomeruli but also transiently in spleen, lymph nodes and thymus of these animals (unpublished observations). These apoptotic bodies are phagocytosed by adjacent tissue cells, as observed in lymphoid organs. The influx of inflammatory cells, observed in the glomeruli at 24 h, and the absence of apoptotic bodies after 24 h indicate that these polymorph nuclear cells (PMN's) might contribute to the clearance of the apoptotic mesangial cell debris. Release of lysozomal enzymes by these PMN's might play a role in the observed increased glomerular permeability. Moreover, the presence of some ER4 deposits, together with C3 and C9 in the glomerular basement membrane (Fig. 2), might also contribute to the increased glomerular permeability.

In this model no autologous phase was observed. A similar finding was previously reported using polyclonal anti-thymocyte serum for

injection into rats [9]. In the latter model polyclonal rabbit IgG could be detected up to 6 weeks after i.v. injection. In the present glomerulonephritis mouse IgG is no longer observed after the 4th day explaining the absence of an autologous phase. The presence of rat IgG observed in some glomeruli after the first week, is confined to those glomerular areas in which extensive fibrin formation is present, and probably reflects non-specific trapping of rat IgG.
The present glomerulonephritis, induced by a single injection of heterologous Moabs, offers a good opportunity to study factors influencing mesangial cell proliferation and mesangial functioning in the rat.

Immunoregulation in AICN

In AICN the presence of cells specifically responding to the disease inducing antigen (Fx1A) have been reported by several authors including ourselves [17, 82, 111, 112]. In addition, cells recognizing antibodies against Fx1A were found leading to the hypothesis that these cells might play a role in immunoregulation in AICN. Using antibodies against the pathogenetic antigen of Heymann nephritis (GP 330) we established the presence of anti-GP 330 recognizing cells in normal animals, and an increased number of these cells after induction of AICN. Higher numbers of anti-GP 330 recognizing cells were present in strains relatively resistant to the induction of AICN. Lower numbers were present in strains being high-responders for the induction of AICN. The data presented in chapter 2 led us to propose the following hypotheses. Firstly, anti-GP 330 recognizing cells possess a GP 330-like antigen as an idiotypic structure of the T-cell antigen receptor and secondly, these cells might be suppressor cells. The presence of suppressor cells has been confirmed in several experimental auto-immune diseases, including AICN [26, 125, 129, 145]. Suppressor cells were observed both after induction of AICN, as well as after induction of high dose tolerance by immunization with the antigen in incomplete Freunds adjuvants [46, 84, 85, 113]. Though in earlier reports these cells were only found in the spleen and lymph nodes, later experiments confirmed their presence in the thymus of rats, both following induction of AICN or following induction of high dose tolerance [46, 84, 85].

In the normal naive animals suppressor cells are known to reside in the thymus. This has clearly been shown in experimental situations resulting in increased incidence of auto-immune phenomena in thymectomized animals. The increased presence of specific suppressor cells in the thymus after disease induction is more difficult to explain. Though there are some reports of immunocompetent cells homing in the thymus [125] immunocompetent cells are thought to migrate out of the thymus and not into it.

If increased numbers of such cells are present in the thymus, these probably arise from local proliferation. The increased incidence of

thymic suppressor cells has been observed in other systems as well. These cells might be precursors leading to progeny migrating to the spleen to exert their suppressive function. It is known that in the rat systemic antigen can induce an increase in large low density cells in the thymus (T1) cells, migrating directly to the spleen, besides the normal traffic of T2 (Mature) cells [61]. However, these cells might also exert a regulatory function only in the thymus.

Though we are aware that the evidence is small that the GP 330 bearing thymocyte is the antigen specific suppressor cell of AICN, the following hypothesis proposed by Herbert and Watson [87] offers a possible explanation combining its suppressor cell nature with idiotypic recognition.

Normally there is an equilibrium between the cells bearing the GP 330 -like idiotype and the cells with the GP 330 antigen receptor. If through immunization this equilibrium is disturbed, the amount of GP 330-like idiotype bearing thymocytes is not sufficient to regulate (suppress) the cells with the GP 330 antigen receptor. These cells proliferate and might become helper cells stimulating anti-GP 330 forming B-cells. (Since these helper cells recognize the GP 330 antigen they might possess idiotype on their antigen specific receptor resembling idiotypes present on anti-GP 330 antibodies. This would explain the observation of Ebert [62] that anti-idiotypic antibodies raised against Fx1a-antibodies suppress the immune response in AICN rats. However, a direct interaction of these anti-idiotypic antibodies on the B-cells can not be excluded.)

If the cells with a GP 330-like idiotype are no longer recognizing the cells with the GP 330 antigen receptor, they might also proliferate, increase in number and thus regain their regulating activity. This leads to reinstalment of the steady state in which higher numbers of GP 330 -like idiotype bearing thymocytes are observed. Upon regaining their regulatory function they might bind cells with the GP 330 antigen receptor, preventing them to migrate and thus leading to diminishing antibody production.

The hypothesis above explains clearly the higher number of GP 330 bearing thymocytes observed in AICN rats coincident with the decrease in antibrushborder production. In addition, it explains why breakthrough of tolerance in strains with higher numbers of GP 330 bearing thymocytes is only achieved by increasing the amount of the

disease inducing antigen. {We and others [45] have observed that repeated immunization of rats of the Brown Norway strain does induce AICN.} Confirmation of this hypothesis will need isolation of this GP 330 bearing cell. Since the number of these cells is small, isolation will prove difficult. The use of monoclonal anti-GP 330 abs might overcome some of these problems.

The role of GP 90 and GP 330 antigens in Heymann nephritis

In both AICN and HICN antibrushborder antibodies play a role and subepithelial immune complexes are observed in the glomerular capillary wall (GCW), in which the presence of anti-brushborder antibodies (a-BB) has been confirmed by elution studies. Our observation that two distinct a-BB specificities can be found in heterologous anti-Fx1A Abs, but only one a-BB specificity in circulating anti-BB abs in AICN, suggested that AICN and HICN might be caused differently. Purification of the heterologous antibodies indicated that the two distinct specificities were directed against GP 90 and GP 330. This thesis focuses on the role of the anti-GP 90 abs in HICN.

Abs eluted from kidney sections of rats with HICN contained large amounts of anti-GP 330 abs as well as low amounts of anti-GP 90 abs. This was supported by Ronco [139] showing the presence of anti-GP 90 Abs in glomerular eluates from HICN rats by immuno-precipitation. These antibodies may be trapped non-specifically in the immune aggregates [25] or may play an active role in the localization of anti-GP 330 Abs in HICN.

Immunohistology at the light microscopic and ultrastructural level as well as ex vivo perfusion studies indicated the presence of GP 90 in the GCW. Recently the GP 90 antigen was identified on both endothelial and epithelial cells in the rat glomerulus by monoclonal abs [43, 139].

Localization of anti-GP 90 abs in vivo was evaluated by injecting rats with these abs. No localization was observed using rabbit anti-GP 90 abs unless very high concentrations of 2 mg were used leading only to a faint staining. Using Moab against GP 90 clear but transient localization in the glomerulus could be observed. The difference in localization using rabbit-anti-GP 90 abs or Moab anti -GP 90, must be explained by the lower amount of specific anti-GP 90 abs in these

rabbit abs compared to Moab anti-GP 90. In addition, most of these anti-GP 90 abs do not reach the kidney because they are bound to GP 90 antigens expressed on several other tissues (liver, spleen, lung, etc) [44, 139]. Experiments by Assmann [7] with polyclonal liver eluted anti-GP 90 abs indicated homogenous (= fine granular) staining 4 hours after i.v.injection of these anti-GP 90 abs into the rat and mouse without subepithelial aggregate formation.

In other species GP 90 abs seem to play a more prominent role. Assmann observed that in the mouse abs, containing both anti-GP 330 and anti-GP 90, induced membranous nephritis, but only in the autologous phase [6]. Since GP 330 is not present on mouse glomerular epithelial cells, these membranous deposits must be initiated by anti-GP 90 abs [8]. Therefore,the possibility exists that anti-GP 90 abs play a role in the formation of immune complexes (Icxs) in HICN. As reported perfusion of polyclonal anti-GP 330 abs, in contrast to intravenous administration, does not lead to localization in the GCW. Iv injection of heterologous antibodies against purified GP 330 performed by Kerjaschki resulted in subepithelial immune complexes [106]. In addition, rats immunized with purified GP 330 developed fine granular deposits [105]. This was recently confirmed by Ronco [142], using affinity purified GP 330 which upon immunization of rats induced typical granular deposits and proteinuria (though no subepithelial deposits were mentioned). Although i.v. administration of mixtures of monoclonal anti-GP 330 abs or polyclonal affinity purified GP 330 abs produced granular deposits, subepithelial deposits were only observed using these polyclonal abs [5]. These data clearly indicate that anti-GP 90 abs are not necessary for Icx formation in HICN.

The finding that anti-GP 330 abs do not bind in the GCW after perfusion, in contrast to the almost immediate localization after i.v.injection, is somewhat surprising. Therefore, we tested whether combinations of anti-GP 90 and anti-GP 330 abs might influence the localization. Combined perfusion or alternating perfusion of polyclonal anti-GP 330 and monoclonal anti-GP 90 did not result in localization of anti-GP 330 in this system. Previous i.v.injection with polyclonal (non-localizing) anti-GP 90 abs or Moab anti-GP 90, followed by perfusion with polyclonal anti-GP 330, did not show localization of anti-GP 330 Abs. From these data it is concluded that the granular subepithelial deposits observed in the rat in HICN are

caused by anti-GP 330 abs, and that anti-GP 90 abs are not necessary for and do not influence localization of anti-GP 330 abs.

Since perfusion of anti-GP 330 abs does not result in localization along the GCW and perfusion of anti-GP 90 does, it was argued that glomerular localization of Icxs after perfusion of heterologous anti-Fx1A antibodies [57] was due to binding of anti-GP 90 abs in the GCW. For this reason the possibility remains open that Icxs containing anti-GP 330 are deposited in the GCW from the circulation. However, there are few data in favor of deposition of circulatory immune complexes in HICN. The presence of GP 330 antigens in the circulation of normal rats is controversial [128] and passive administration of preformed Fx1A-anti-Fx1A immune complexes does not lead to subepithelial Icx formation [57]. Moreover, the presence of the GP 330 epitope on the epithelial cell has been proven and is a strong argument in favor of the in situ formation [8, 43, 105, 106].

In view of the recently developed hypothesis of the role of crosslinking of antibodies for the induction of granular immune deposits in other antigen-antibody systems [24, 117], combined with the different results obtained using monoclonal anti-GP 330 abs or polyclonal anti-GP 330 abs [5], the concept of crosslinking offers an elegant explanation for the formation of subepithelial Icx deposits in HICN and AICN. In HICN rabbit anti-GP 330 abs after i.v.injection might cross the GCW (mechanism unknown), recognize the GP 330 antigen on the epithelial cell and bind there in a confluent pattern. Only if higher amounts of anti-GP 330 abs (with the ability to crosslink) have traversed the GCW, crosslinking of these abs might occur, leading to `capping' and `shedding' of these Icxs. This leads to subepithelial Icx deposits, first observed by EM and later by IF.

Though at the moment few data have been presented proving such a hypothesis, the in vitro experiments by Camussi et al [35] indicate that cultured epithelial cells do bind anti-GP 330, leading to a process of capping and shedding of the antigen-antibody complex. The absence of subepithelial deposits after injection of anti-GP 330 abs in rats in which "capping" of the epithelial cell was prevented by treatment with chlorpromazine, suggests that crosslinking, capping and shedding of Icxs might also play a role in vivo [36].

The hypothesis of crosslinking necessary to induce granular subepithelial immune complex deposits is very interesting, as this

hypothesis might explain various, so far confusing, findings in HICN. Firstly, after ex vivo perfusion of anti-GP 330 abs the low amount of bound anti-GP 330 as well as the rather short perfusion time is not enough to allow for the crosslinking. Thus no granular Icx deposits are observed. Perfusion studies using much longer perfusion times might enable enough anti-GP 330 abs to localize, crosslink and lead to capping of these complexes. After capping these complexes might become visible first as subepithelial deposits in EM microscopy and later as granular deposits in the IF microscopy techniques. Although we must bear in mind that perfusion studies with eluted abs of rats with AICN might posses anti-GP 90 abs [104], the linear localization of rat IgG in this system observed with IEM might thus be explained as well as the few subepithelial deposits [57, 93]. Secondly, the pattern of small, slowly increasing Icx deposits seen after i.v.injection of anti-GP 330 abs and reaching a maximum after 4 days [147] might also be explained by the gradual accumulation and shedding of Icxs. Thirdly, the absence of granular deposits after i.v.injection of Fab fragments of sheep anti-FxlA IgG might be due to their inability to crosslink [148]. Fourthly, the absence of localization of autologous [53] and heterologous [28, 146] anti-brushborder abs in rats treated with aminonucleoside of puromycin (PAN), might be explained by alteration of the epithelial cell. Although no decrease in the epithelial expression of FxlA antigens was observed, epithelial function alters probably affecting membrane fluidity and crosslinking.

Anti-thymocyte antibodies

Our interest in antibodies reacting with thymocytes began when we discovered dual specificity in heterologous anti-FxlA antibodies i.e. anti-BB and anti-thymocyte specificity. The question was raised whether immunization of rabbits with thymocytes might result in antibodies with such a dual specificity. Although this was the case, i.e we observed anti-brushborder activity in these abs, probably directed against the GP 90 antigen, it became soon evident that also other specificities were involved. Especially the mesangial staining of these antibodies suggested the recognition of another antigen, the THY 1 antigen [97, 136].

In general, the Thy 1 antigen is an intriguing molecule. (See reference 55 for a review). It was discovered in 1964 and it is a

glycoprotein with a MW of 25 kD. Despite a detailed knowledge of its structure, the function still remains to be determined. The Thy-1 antigen (or homologues) are present in many species (mouse, dog, rat, human) although its distribution on the various tissues of each species differs. In all species, however, the antigen has been found on the brain. The presence of Thy 1 in the glomerulus has been reported only in the rat [123].

The presence of the Thy 1 antigen in the rat mesangium as well as in the rat GBM and not in the tubular basement membrane excludes a crossreaction of anti-Thy 1 Moabs with laminin, fibronectin, actin, entactin and types of collagen. Deposition from the circulation is not very likely. Circulating Thy1 antigens in the rat are only present in very low (nanogram) amounts [123]. This is supported by the observation that i.v.injection of Moab anti-Thy1 does not lead to deposition in the liver. Neonatal rats posses already substantial amounts of Thy 1 antigen on their mesangium. The most convincing evidence however is the presence of the Thy1 antigen on the cell membrane of cultured mesangial cells [124].

The nephritis observed after injection of monoclonal anti-Thy 1 antibodies is initiated by the direct binding of these antibodies to the rat mesangium and GBM. The activation of complement (C3) and especially that of C9 indicates the involvement of Membrane Attack Complex (MAC). Although the exact deleterious mechanism of MAC is unknown, it might generate O2- radicals toxic for the mesangial cell through influx of inflammatory cells [4]. In addition, it might play a role in the proteinuria observed [137]. The role of complement is in agreement with studies from Wilson [179] showing that complement depletion by Cobra Venom factor abolishes glomerulonephritis in rats induced by polyclonal anti-Thy1.1 antibodies.

The lysis of the mesangial cell is clearly shown by light and electron microscopy and seems more an event of apoptosis than of necrosis [152]. The polymorph nuclear cells attracted by complement and observed at the first hours after i.v. injection might play a role in the clearing of the mesangial cell debris. The absence of mesangial cell contraction and loosening of the anchoring points of the GBM leads to the formation of capillary aneurysms. Presence of fibrinogen indicates concomitant activation of the coagulation cascade. Mesangiolysis is followed by increased proliferation of mesangium

cells, probably from locally surviving cells. The agent inducing proliferation is at the moment unknown. The data from the group of Karnovsky in the Habu Snake model in the rat indicated that both in vitro [38] and in vivo [49], heparin might play a role in this process, although platelet derived growth factor might also contribute to the proliferation of these cells [124]. Not only proliferation of mesangial cells, but also proliferation of epithelial cells of Bowman's capsule is observed, leading to the formation of crescents. In concordance with the crescent formation observed in nephrotoxic serum nephritis, the fibrin depositions might initiate these events [175]. All these histological alterations diminish, and after 10 weeks only mesangial cell hypercellularity is observed. The relatively benign course of this nephritis might be related to the transient localization of mouse anti-Thy 1 antibodies. This might be overcome by using a telescoped model i.e. preimmunization of the rat with mouse IgG. All in all, this model offers a good opportunity to study the origin of newly proliferating mesangial cells, as well as the influence of increased mesangial hypercellularity on mesangial function (i.e. uptake of macromolecules).

Relation of animal models to human forms of glomerulopathy.

The membranous nephritis observed in AICN and HICN are assumed counterparts of human membranous glomerulopathy. Since in both AICN and HICN antibodies recognizing determinants of the rat kidney tubular brushborder are involved, a search was started for anti-brushborder antibodies either in the circulation or in the glomerular deposits in human idiopathic membranous glomerulopathy. So far, only conflicting data exist. Naruse [126] and Droz [60] were able to show Fx1A antigens in respectively 3 out of 7 and 8 out of 28 human kidney biopsies of patients with idiopathic membranous nephritis. This could not be confirmed by Whitworth [174] studying 16 biopsies of idiopathic membranous patients, or Collins [50] screening 68 renal biopsies of patients with idiopathic membranous glomerulonephritis. Moreover, circulatory anti-Fx1A abs in these patients were only sporadically found [58, 115]. Since the GP 330 antigen is only found in rat glomerular epithelial cells, a search for anti-GP 330 abs in the human seems not warranted.

The mesangial cell proliferation observed in the nephritis induced by anti-Thy 1 antibodies might bear relation to human forms of glomerulopathy. If in the human situation antibodies are formed reacting with mesangial cells, lysis of these cells might take place. This would go unnoticed, as in general clinical symptoms of nephritis are not immediately followed by kidney biopsies. Since the lysis of the mesangial cell is readily followed by proliferation, the diagnosis of mesangial hypercellularity would be the only pathological finding. A search for antibodies reacting with the mesangium in the sera of such patients (especially just after the start of the nephritis) might give insight whether antibodies against mesangial cells play a role in human forms of mesangial hypercellularity.

So far, the use of extensively absorbed polyclonal anti-thymocyte antisera in treatment of human rejection of kidney grafts has indicated that these antibodies generally do not cause nephritogenicity [94]. It remains a good precaution to test antibodies for the presence of unwanted anti-kidney antibodies before applying them in allograft rejection. Since monoclonal anti-T cell antibodies do not react with antigens located in normal human glomeruli [138] the use of anti-T cell Moabs seems without hazards [166].

SUMMARY

Mechanisms of immune complex formation and localization have been studied in experimental models. Considerable interest has been paid to the hazards of immune complexes in the kidney, formed in situ or deposited from the circulation.

In this thesis we describe two experimental models, which are assumed counterparts of human membranous nephritis, i.e. the autologous (AICN) and heterologous immune complex glomerulonephritis (HICN). In both models antibodies (abs) are present, directed against brushborder antigens on the proximal tubule of the rat kidney. These abs have nephritogenic properties due to their ability to recognize an antigen on the glomerular epithelial cell. Besides humoral immunity due to antibrushborder abs, the impact of cellular immunity for regulation of the immune response has increased in these models.

In chapter 2 we present a possible model for the regulation through specific antigen recognizing cells and antibody recognizing cells in AICN. Chapters 3,4,5, and 6 describe the different specificities observed in heterologous anti-brushborder antibodies, i.e the characterization of the distinct specificities, the characterization of the determinants to which they are directed, and the contribution of each specificity to Icx localization in HICN. The observation of antibodies reacting with thymocytes in anti-brushborder abs directed our interest to the nephritogenic potential of antibodies reacting with thymocytes. These data are presented in chapter 7,8,9 and 10.

Chapter 2 describes the numbers of thymocytes reacting with an antibody directed to the pathogenetic antigen of Heymann nephritis, i.e. GP 330, as observed by immuno-fluorescence microscopy (IF). It is shown that in animals with AICN a decrease in circulatory anti-Fx1A abs coincides with an increase in the number of GP 330 bearing thymocytes. In animals relatively resistant for induction of AICN, relatively more GP 330 bearing thymocytes are observed than in animals of susceptible strains. The hypothesis is put forward that these GP 330 bearing cells are suppressor cells regulating the immune response in AICN through an antigen receptor possessing an idiotype resembling GP 330.

Chapter 3 describes the observation of dual specificity in both anti-brushborder antibodies as well as in rabbit- anti-thymocyte antibodies, as shown by respectively cytotoxicity, IF and MIF-assays.

In Chapter 4 we describe that antibodies eluted from kidneys of rats with HICN possess both anti-brushborder as well as anti-T specificity. This indicates a possible role for these anti-T specificities in Icx formation in HICN. Immunoabsorption with rat heart tissue removed the anti-T specificity, leaving only anti-brushborder abs that by immunoblotting are shown to be directed against an antigen with a MW of 330 kD. The anti-T -cell antibodies were eluted from the rat heart tissue used for immuno-absorption. Immunoblotting showed that these abs recognized an antigen with a MW of 90 kD. Perfusion (IF) and in vitro incubation experiments (IF and IEM) on kidney sections indicated that localization along the glomerular capillary wall (GCW) was only due to the anti-GP 90 specificities in anti-Fx1A antibodies.

However, the literature claimed the GP 330 antigen to be the pathogenetic antigen in Heymann nephritis. Therefore, we compared our findings of polyclonal anti-Fx1A antibodies with monoclonal antibodies against GP 330 and GP 90, and with polyclonal antibodies directed against purified GP 330. Comparison of the immunohistological staining patterns of these antibodies on lymphoid organs indicated that the anti-T specificity recognized the same antigen as anti-GP 90 Moab. These results confirmed the earlier immunoblotting experiments and are presented in chapter 5.

A possible role for ischaemia in the localization of antibodies was excluded by repeating the perfusion experiments in the controlled kidney perfusion system. The results were the same, i.e. no localization of anti-GP 330 antibodies and clear localization of anti-GP 90 antibodies or anti-Fx1A abs. Intravenous injection of small amounts of anti-GP 330 abs showed localization along the GCW. Iv injection of anti-GP 90 abs did show localization, but considerable amounts were needed. A role of anti-GP 90 abs in the localization of anti-GP 330 antibodies was excluded by alternating perfusion and perfusion of mixtures of these abs. Systemic administration of polyclonal and monoclonal anti-GP 90 antibodies also did not cause localization of anti-GP 330 antibodies after subsequent perfusion. These data exclude a role for anti-GP 90 abs and confirm the role of anti-GP 330 abs in ICx in HICN. The absence of detectable localization

of anti-GP 330 after perfusion ex vivo might be explained by the need to crosslink of these antibodies. Arguments in favor of such a mechanism are presented in chapter 6 .

Heterologous antibodies against thymocytes were made to compare this anti-thymocyte activity with the anti-T activity in anti-Fx1A. It soon became evident that we were dealing with two different anti-T specificities. Although carefully absorbed anti-thymocyte antibodies bind to the rat kidney both in vitro and after iv injection, the binding pattern was distinctly different from the one observed with anti-Fx1A antibodies. Furthermore, no nephritis was observed using these heterologous anti-thymocyte antibodies. These results are presented in chapter 7.

The primarily mesangial localization indicated involvement of anti-Thy 1 antibodies, since the Thy 1 antigen is shared between rat thymocyte membranes and rat mesangium. Using monoclonal anti-Thy 1 antibodies, we investigated to which component of the rat glomerulus these monoclonal anti-Thy-1 Abs were directed. It was shown both by IF and IEM, in vitro and after perfusion ex vivo, that these abs recognized an antigen not only in the rat mesangium but also in the rat GCW. These results are presented in chapter 8.

In vivo administration of relatively low amounts of anti-Thy 1 Moabs immediately showed localization of these Moabs in the rat kidney. The induction of a fulminant, but transient, nephritis is shown to be dependent on the use of complement activating subclasses of these Moabs. This nephritis is initiated by the direct binding of these abs to the rat mesangium and GBM, followed by complement activation, and mesangial changes leading to capillary aneurysms. Mesangial cell proliferation, proliferation of cells of Bowman's capsule, and interstitial infiltrates are observed at day 14. The height of the proteinuria and the main histological alterations observed in the first 14 days after iv injection are described in chapter 9.

At the ultrastructural level lysis of the mesangial cells seems the underlying pathogenetic mechanism. This leads to detachment of the anchoring points of the GBM in the non-existent mesangium, resulting in capillary aneurysms, followed by mesangial cell proliferation. Mouse IgG disappears rapidly. In this model the proteinuria as well as the nephritis is transient. At week 10 only mesangial hypercellularity can be observed. Although at the ultrastructural level collagenous

fibers are observed in the mesangium, there is no histological evidence of sclerosis. The findings in this model resemble those observed after injection of Habu Snake Venom. This unique form of nephritis is described in chapter 10.

SAMENVATTING

De mechanismen van immuun-complex formatie en localisatie worden in experimentele modellen bestudeerd. Veel aandacht is besteed aan de risico's van immuun-complexen in de nier, in situ gevormd of vanuit de circulatie afgezet.

In dit proefschrift worden twee experimentele modellen beschreven, waarvan aangenomen wordt dat zij pendanten zijn van humane membraneuze nephritis: de autologe (AICN) en heterologe (HICN) immuun-complex glomerulonephritis. In beide modellen zijn antilichamen aanwezig, gericht tegen antigenen van de borstelzoom van de proximale tubulus van de rattenier. Deze antilichamen hebben nephritogene eigenschappen vanwege hun vermogen om een antigeen op de glomerulaire epitheelcel te herkennen. Naast humorale immuniteit vanwege borstelzoom antilichamen wordt aan cellulaire immuniteit voor de regulatie van de immuun respons een steeds grotere rol toegekend in deze modellen.

In hoofdstuk twee wordt een mogelijk model aangeboden voor de regulatie via specifieke antigeen herkennende cellen en antilichaam herkennende cellen in AICN.

De hoofdstukken 3, 4, 5 en 6 beschrijven de specificiteiten aanwezig in heterologe anti-borstelzoom antilichamen,dwz. de karakterisatie van de verschillende specificiteiten, de karakterisatie van de determinanten waar zij tegen gericht zijn, en de bijdrage van elke specificiteit aan immuun-complex localisatie in HICN.

Vanwege de aanwezigheid van anti-thymocyten antistoffen in heterologe anti-borstelzoom antistoffen werd de nephritogeniciteit van anti-thymocyten antistoffen onderzocht. De gegevens hierover worden vermeld in de hoofdstukken 7, 8, 9 en 10.

Hoofdstuk twee beschrijft de aantallen thymocyten die reageren met een antilichaam dat gericht is tegen het pathogenetische antigeen van de Heyman nephritis,dwz. GP 330, zoals waargenomen met behulp van immuno-fluorescentie microscopie (IF). Er werd aangetoond dat in dieren met AICN een afname in circulerende anti-FxlA antilichamen samenvalt met een toename in het aantal GP 330 dragende thymocyten. In dieren die relatief resistent zijn voor de inductie van AICN worden naar verhouding meer GP 330 dragende thymocyten waargenomen dan in

voor AICN gevoelige stammen. De hypothese wordt aangedragen dat deze GP 330 dragende cellen suppressor cellen zijn die de immuun-respons in AICN reguleren door middel van een antigeen receptor die een idiotype draagt dat lijkt op GP 330.

Hoofdstuk 3 beschrijft de waarneming van tweeledige specificiteit in zowel konijne-anti-borstelzoom antilichamen als ook in konijne- anti-thymocyten antilichamen, aangetoond met behulp van respectivelijk: cytotoxiciteit, IF en MIF-assays.

In hoofdstuk 4 wordt beschreven dat antilichamen die ge-elueerd zijn uit nieren van ratten met HICN zowel anti-borstelzoom als anti-T specificiteit bezitten. Dit duidt op een mogelijke rol voor deze anti-T specificiteiten in immuun-complex formatie in HICN. Immuno-absorptie met rattehart weefsel verwijderde de anti-T specificiteit, waarna alleen anti-borstelzoom antilichamen overbleven, waarvan met behulp van immunoblotting bleek dat ze gericht waren tegen een antigeen met een MW van 330 kD. De anti-T cel antilichamen werden ge-elueerd uit rattehart weefsel dat gebruikt was voor immuno-absorptie. Immunoblotting toonde aan dat deze antilichamen een antigeen herkenden met een MW van 90 kD. Perfusie (IF) en in vitro incubatie experimenten (IF en IEM) op stukjes nier toonde aan dat localisatie langs de glomerulaire capillaire wand (GCW) alleen veroorzaakt werd door de anti-GP 90 specificiteit in anti-Fx1A antilichamen.
Volgens de literatuur echter was GP 330 het pathogenetische antigeen in Heyman nephritis. Daarom werden polyclonale anti-Fx1A antilichamen vergeleken met monoclonale antilichamen (Moab) tegen GP 330 en GP 90 en met polyclonale antilichamen gericht tegen gezuiverd GP 330. Vergelijking van de immuno-histologische kleuringspatronen van deze antilichamen op lymphoide organen gaf aan dat de anti-T specificiteit hetzelfde antigeen herkende als anti-GP 90 Moab. Deze resultaten bevestigden de eerdere immunoblotting experimenten en worden vermeld in hoofdstuk 5.

Een mogelijke rol voor ischaemia in de localisatie van antilichamen werd uitgesloten door de perfusie experimenten te herhalen in het gecontroleerde nier perfusie systeem. De resultaten waren dezelfde: dwz. geen localisatie van anti-GP 330 antilichamen en een duidelijke

localisatie van anti-GP 90 en anti-FxlA antilichamen. Intraveneuze (iv) injectie van kleine hoeveelheden anti-GP 330 leidde tot localisatie langs de GCW. Iv injectie van anti-GP 90 antilichamen vertoonde slechts dan localisatie wanneer aanzienlijke hoeveelheden gebruikt werden. Een rol van anti-GP 90 antilichamen in de localisatie van anti-GP 330 antilichamen werd uitgesloten door alternerende perfusie en perfusie van mengsels van deze antilichamen. Systemische toediening van polyclonale en monoclonale anti-GP 90 antilichamen gevolgd door perfusie van anti-GP 330 veroorzaakte geen localisatie van deze antilichamen. Deze gegevens sluiten een rol uit voor anti-GP 90 antilichamen en bevestigen de rol van anti-GP 330 antilichamen in ICx vorming in HICN. De afwezigheid van aantoonbare localisatie van anti-GP 330 na perfusie ex vivo zou kunnen betekenen dat deze antilichamen moeten crosslinken. Argumenten voor zo'n mechanisme worden in hoofdstuk 6 gegeven.

Heterologe antilichamen tegen thymocyten werden gemaakt om deze anti-thymocten activiteit te vergelijken met de anti-T activiteit in anti-FxlA. Spoedig bleek dat hier sprake was van twee verschillende anti-T specificiteiten. Hoewel zorgvuldig geabsorbeerde anti-thymocyten antilichamen binden aan de rattenier, zowel in vitro als na iv injectie, was het bindingspatroon duidelijk anders dan die welke aangetoond was met anti-FxlA antilichamen. Bovendien leidden deze heterologe anti-thymocyten antilichamen niet tot een nephritis. Deze resultaten worden beschreven in hoofdstuk 7.

De primaire mesangiale localisatie duidde op betrokkenheid van anti-Thy 1 antilichamen, aangezien het Thy 1 antigeen aanwezig is op zowel de thymocyten membranen als het mesangium van de rat. Met behulp van monoclonale anti-Thy 1 antilichamen werd onderzocht tegen welkecomponent van de ratteglomerulus deze monoclonale anti-Thy 1 antilichamen waren gericht. Er werd aangetoond zowel met behulp van IF en IEM, in vitro en na perfusie ex vivo, dat deze antilichamen een antigeen herkenden dat in het mesangium en in de GCW van de rat aanwezig was. Deze resultaten worden beschreven in hoofdstuk 8.

In vivo toediening van relatief kleine hoeveelheden anti-Thy 1 Moabs vertoonde onmiddelijk localisatie van deze Moabs in de rattenier. De

inductie van een fulminante, maar tijdelijke, nephritis bleek afhankelijk te zijn van het gebruik van complement activerende subklassen van deze Moabs. Deze nephritis wordt geinitieerd door de directe binding van deze antilichamen aan het mesangium en de GBM van de rat, gevolgd door activering van complement, en mesangiale veranderingen die leiden tot capillaire aneurysmata. Mesangiale celproliferatie, proliferatie van de cellen van het kapsel van Bowman, en interstitiele infiltraten worden op dag 14 waargenomen. De hoogte van de proteinurie en de belangrijkste histologische veranderingen waargenomen tijdens de eerste 14 dagen na iv injectie worden in hoofdstuk 9 beschreven.

Op het ultrastructurele niveau blijkt lysis van de mesangiale cel het onderliggende pathogenetische mechanisme te zijn. Dit leidt tot loslating van de ankerpunten van de GBM in het niet meer bestaande mesangium, hetgeen leidt tot capillaire aneurysmata, gevolgd door mesangiale celproliferatie. Het muis IgG verdwijnt snel. In dit model zijn zowel de proteinurie als de nephritis tijdelijk. Na 10 weken wordt slechts mesangiale hypercellulariteit waargenomen. Hoewel op het ultrastructurele niveau collagene vezels worden waargenomen in het mesangium is er geen histologisch bewijs van sclerose. De bevindingen in dit model lijken op die welke waargenomen zijn na injectie van Habu Snake Venom. Deze unieke vorm van nephritis wordt beschreven in hoofdstuk 10.

1. Abdou NI. Generation of suppressor cells by administration of deaggregated horse anti-human thymocyte globulin in man. Transplant Proc 1981; 13: 512.
2. Abrahamson DR & Caulfield JP. Proteinuria and structural alterations in rat glomerular basement membranes induced by intravenously injected anti-laminin immunoglobulin G. J Exp Med 1982; 156: 128.
3. Abrass CK, Border WA & Glassock RJ. Circulating immune complexes in rats with autologous immune complex nephritis. Lab Invest 1980; 43: 18
4. Adler S, Baker PJ, Johnson RJ, Ochi RF, Pritzl P & Couser WG. Complement Membrane Attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. J Clin Invest 1986; 77: 762
5. Allegri L, Ferrari C, Manara GC, Brianti E, Chatelet F, Dall'Aglio PP, Ronco P & Verroust P. Antigen antibody interactions in Heymann nephritis (HN) Kidney Int 1986; 29: 608 (abstract)
6. Assmann KJM, Tangelder MM, Lange WPJ, Tadema TM & Koene RAP. Membranous glomerulonephritis in the mouse. Kidney Int 1983; 24: 303
7. Assmann KJM, Ronco P, Tangelder MM, Lange WPH, Verroust P & Koene RAP. Involvement of an antigen distinct from the heymann antigen in membranous glomerulonephritis in the mouse. Academic Thesis, Nijmegen 1985, Chapter 4.
8. Assmann KJM, Lange WPH, Tangelder MM & Koene RAP. The organ distribution of gp-330 (Heymann antigen) and gp-90 in the mouse and the rat. Virchows Arch [Pathol Anat] 1986; 408: 541
9. Bagchus WM, Donga J, Hoedemaeker PhJ, & Bakker WW. The specificity of nephritogenic antibodies. II. Immune complex glomerulopathy in rats induced by heterologous antithymocyte serum. Transplantation 1984; 38: 165.
10. Bagchus WM, Vos JTWM & Bakker WW. Anti-T-cell specificity and localization of heterologous nephritogenic antibodies in the rat. Kidney Int 1984; 25: 732 (abstract).
11. Bagchus WM, Vos JTWM, Rozing J, Hoedemaeker PhJ & Bakker WW. Anti-thymocyte antibodies and kidney antigens. Kidney Int 1985; 27: 703 (abstract)
12. Bagchus WM, Donga J, Rozing J, Hoedemaeker PhJ & Bakker WW. The specificity of nephritogenic antibodies. IV Binding of monoclonal anti- thymocyte antibodies to rat kidney. Transplantation 1986; 41: 745
13. Bagchus WM, Rozing J, Hoedemaeker PhJ & Bakker WW. Acute glomerulonephritis after intravenous injection of monoclonal anti - thymocyte antibodies in the rat. Immunology Letters 1986: 12:109
14. Bagchus WM, Vos JTWM, Hoedemaeker PhJ & Bakker WW. The specificity of nephritogenic antibodies. III.Binding of anti-Fx1A antibodies in glomeruli is dependent on dual specificity. Clin exp Immunol 1986; 63: 639
15. Bakker WW, Engelhart JJ, Mulder I & Hoedemaeker PhJ. Migration inhibitory factor(s) from specifically stimulated thymocytes in vitro. Int Arch Allergy 1975; 49: 491.
16. Bakker WW, Mulder I, vd Lee RJ, Fleuren GJ & Hoedemaeker PhJ. Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens: III Cell mediated immune response against immune complexes influenced by immunosuppressive therapy. Int Arch Allergy 1977; 54: 695

17. Bakker WW, Kooistra K, Vos JTWM & Hoedemaeker PhJ. Suppressor cells directed against self receptors in experimental autologous glomerulonephritis detected by MIF-assays.In: Cell biology and Immunology of Leukocyte function, 1979, pp 695, Academic Press, New York-San Francisco-London.
18. Bakker WW, Bagchus WM & Hoedemaeker PhJ. Antithymuscell activity of nephritogenic antibodies. Transplantation 1979; 27: 217
19. Bakker WW, Bagchus WM, Vos JTWM, Kooistra K, & Hoedemaeker PhJ. The specificity of nephritogenic antibodies: I.Evidence on anti-T-cell specificity in nephritogenic antibodies detected by cytotoxicity and MIF-assays. Immunobiol 1981; 159: 235.
20. Balch Ch & Feldman JD. Thymus-dependent (T) lymphocytes in the rat. J Immunol 1974; 112: 79.
21. Barabas AZ & Lannigan R. Induction of an autologous immune complex glomerulonephritis in the rat by intravenous injection of heterologous anti-rat kidney tubular antibody. I. Production of chronic progressive immune-complex glomerulonephritis. Br J exp Path 1974; 55: 47.
22. Barabas AZ & Lannigan R. Induction of an autologous immune complex glomerulonephritis in the rat by intravenous injection of heterologous anti-rat kidney tubular antibody. II. Early glomerular lesions. Br J exp Path 1974; 55: 282
23. Barabas AZ, Cornish J & Lannigan R. Passive heymann nephritis in the rat produced by a heterologous antibody to a heterologous kidney fraction 3 antigen. Br J exp Path 1982; 63: 667.
24. Barba LM, Caldwell PRB, Downie GH, Camussi G, Brentjens JR & Andres G. Lung injury mediated by antibodies to endothelium I. In the rabbit a repeated interaction of heterologous anti- angiotensin-converting enzyme antibodies with alveolar endothelium results in resistance to immune injury through antigenic modulation. J Exp Med 1983; 158: 2141.
25. Bellon B, Belair MF, Kuhn J, Druet P & Bariety J. Trapping of circulating proteins in immune deposits of Heymann nephritis. Lab Invest 1982; 46: 306.
26. Ben-Nun A & Cohen IR. Spontaneous remission and acquired resistance to autoimmune encephalomyelitis (EAE) are associated with suppression of T cell reactivity: suppressed EAE effector cells recovered as T cell lines. J Immunol 1982; 128: 1450.
27. Bender BL, Jaffe R, Carlin B & Chung AE. Immunolocalization of Entactin, a Sulfated Basement Membrane Component, in Rodent Tissues, and Comparison With GP-2 (Laminin). Am J Pathol 1981; 103: 419.
28. Bertani T, Nolin L, Foidart J, Vandewalle A & Verroust P. The effect of puromycin on subepithelial deposits induced by antibodies directed against tubular antigens: a quantitative study. Eur J of Clin Invest 1979; 9: 465.
29. Bona CA & Paul WE. Cellular basis of regulation of expression of idiotype. I T-suppressor cells specific for MOPC 460 idiotype regulate the expression of cells secreting anti-TNP antibodies baering 460 idiotype. J Exp Med 1979; 149: 592
30. Bona CA, Finley S, Waters S & Kunkel HG. Anti- immunoglobulin antibodies. III. Properties of sequential anti-idiotypic antibodies to heterologous anti-gamma globulins. Detection of reactivity of anti-idiotype antibodies with epitopes of Fc-fragments (homobodies) and with epitopes and idiotopes (epibodies). J Exp Med 1982; 156: 986.
31. Bradfield JWB, Cattell V & Smith J. The Mesangial Cell in Glomerulonephritis II Mesangial Proliferation Caused by Habu Snake Venom in the Rat. Lab Invest 1977; 36: 487.

32. Bruener K, Opitz HG & Koelsch E. The thymus as primary site for antigen-specific T-suppressor cells in neonatally induced tolerance to bovine serum albumin. Immunobiol 1982; 162: 221.
33. Buckley PJ & Dickson SA. Monoclonal antibodies to T-helper/inducer and T-suppressor/cytotoxic lymphocyte subsets recognize antigens on splenic sinusoidal lining cells. Am J Clin Pathol 1984; 82: 167.
34. Busch GJ, Birtch AG, Lukl P, Kobayashi K, Galvanek EG & Carpenter CB. Human renal allografts: glomerular deposits of horse immunoglobulin F and nephritis following administration of anti-lymphocyte globulin. Hum Pathol 1971; 2: 299.
35. Camussi G, Brentjens JR, Noble B, Kerjaschki D, Malavasi F, Roholt OA, Farquhar MG & Andres G. Antibody- induced redistribution of heymann antigen on the surface of cultured glomerular visceral epithelial cells: possible role in the pathogenesis of heymann glomerulonephritis. J Immunol 1985; 135: 2409.
36. Camussi G, Noble B, van Liew J, Brentjens JR & Andres G. Pathogenesis of passive heymann glomerulonephritis: chlorpromazine inhibits antibody- mediated redistribution of cell surface antigens and prevents development of the disease. J Immunol 1986; 136: 2127.
37. Carlsson SV & Stigbrand T. Partial characterization of the oligosaccharides of mouse thymocyte Thy-1 glycoprotein. Biochem J 1984; 221: 379.
38. Castellot JJ, Hoover RL, Harper PA & Karnovsky MJ. Heparin and glomerular epithelial cell-secreted heparinlike species inhibit mesangial-cell proliferation. Am J Pathol 1985; 120: 427.
39. Cattell V, Bradfield JWB. Focal Mesangial Proliferative Glomerulonephritis in the Rat Caused by Habu Snake Venom. Am J Pathol 1977; 87: 511.
40. Cattell V. Focal mesangial proliferative glomerulonephrits in the rat caused by Habu Snake Venom: the role of platelets. Br J exp Path 1979; 60: 201.
41. Cattell V & Mehotra A. Focal mesangial proliferative glomerulonephritis in the rat caused by Habu Venom: the effects of anti- platelet agents. Br J exp Pathol 1980; 61: 310.
42. Chant S, Katz A & Silverman M. Pathogenicity of a highly purified brush border membrane preparation in Heymann nephritis. J Clin Lab Immunol 1980; 4: 133.
43. Chatelet F, Brianti E, Ronco P, Roland J & Verroust P. Ultrastructural localization by monoclonal antibodies of brushborder antigens expressed by glomeruli. I. Renal Distribution. Am J Pathol 1986; 122: 500.
44. Chatelet F, Brianti E, Ronco P, Roland J & Verroust P. Ultrastructural localization by monoclonal antibodies of brushborder antigens expressed by glomeruli. II. Extrarenal Distribution. Am J Pathol 1986; 122: 512.
45. Cheng IKP, Dorsch SE & Hall BM. Lymphocyte subsets in Heymann nephritis. Lab Invest 1984; 51: 286.
46. Cheng IKP, Dorsch SE & Hall BM. Immunoregulation in Heymann's nephritis: modulation by suppressor T cells. Kidney Int 1985; 28: 866 (abstract).
47. Cleveland WL, Wasserman NH, Sarangarajan R, Penn AS & Erlanger BF. Monoclonal antibodies to the acetylcholine receptor by a normally functioning auti-anti- idiotypic mechanism. Nature 1983; 305: 56.
48. Cochrane CC. Mechanisms involved in the deposition of immune complexes in tissues. J Exp Med 1971; 134: 75.

49. Coffey AK & Karnovsky MJ. Heparin Inhibits Mesangial Cell Proliferation in Habu-Venom-Induced Glomerular Injury. Am J Pathol 1985; 120: 248.
50. Collins AB, Andres GA & McCluskey RT. Lack of evidence for a role of renal tubular antigen in human membranous glomerulonephritis. Nephron 1981; 27: 297.
51. Courtoy PJ, Kanwar YS, Hynes RO & Farquhar MG. Fibronectin Localization in the Rat Glomerulus. J Cell Biol 1980; 87: 691.
52. Couser WG, Stilmant MM & Darby C. Autologous Immune Complex Nephropathy I. Sequential Study of Immune Complex Deposition, Ultrastructural Changes, Proteinuria, and Alterations in Glomerular Sialoprotein. Lab Invest 1976; 34: 23.
53. Couser WG, Jermanovich NB, Belok S, Stilmant MM & Hoyer JR. Effect of aminonucleoside nephrosis on immune complex localization in autologous immune complex nephropathy in rats. J Clin Invest 1978; 61: 561.
54. Couser WG, Steinmuller DR, Stilmant MM, Salant DJ & Lowenstein LM. Experimental glomerulonephritis in the isolated perfused rat kidney. J Clin Invest 1978; 62: 1275.
55. Crawford JM & Barton RW. Biology of Disease. Thy-1 glycoprotein: Structure, distribution, and Ontogeny. Lab Invest 1986; 54: 122.
56. Dales S, Fujinami RS & Oldstone MBA. Serologic relatedness between Thy-1.2 and actin revealed by monoclonal antibody. J Immunol 1983; 131: 1332.
57. van Damme BJC, Fleuren GJ, Bakker WW, Vernier RL & Hoedemaeker PhJ. Experimental glomerulonephrits in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. Lab Invest 1978; 38: 502.
58. Douglas MFS, Rabideau DP, Schwartz MM & Lewis EJ. Evidence of autologous immune-complex nephritis. New Engl J Med 1981; 26: 1326.
59. Doxsey S, Kerjaschki D & Farquhar MG. A large membrane glycoprotein (gp-330) is a resident of coated pits of several absorptive epithelia. J Cell Biol 1983; 97: 178 (abstract).
60. Droz D, Noel LH, Fouchard M, Perrone B & Barbanel C. Presence of renal tubular epithelium antigen in glomeruli of membranous glomerulonephritis: An indicator of renal vein thrombosis. Abstr 8th Int Congr Nephrol Athens 1981.
61. Durkin HG, Carboni JM & Waksman BH. Antigen-induced increase in migration of large cortical thymocytes (regulatory cells?) to the marginal zone and red pulp of the spleen. J Immunol 1978; 121: 1075.
62. Ebert TH, Mccluskey RT, Collins AB & Colvin RB. Modulation of autologous immune complex nephritis (AIC) by pre-immunization with autoantibodies or sensitized cells. Kidney Int 1981; 19: 181 (abstract)
63. Edgington TS, Glassock RJ, Watson JI & Dixon FJ. Characterization and isolation of specific renal tubular antigens. J Immunol 1967; 99: 1199.
64. Edgington TS, Glassock RJ & Dixon FJ. Characterization and isolation of specific renal tubular epithelial antigens. J Immunol 1967; 99: 1199.
65. Edgington TS, Glassock RJ & Dixon FJ. Autologous immune complex glomerulonephritis induced with renal tubular antigen. I. Identification and isolation of the pathogenetic antigen. J Exp Med 1968; 127: 555.
66. Edgington TS, Lee S & Dixon FJ. Persistence of the autoimmune pathogenetic process in experimental autologous immune complex nephritis. J Immunol 1969; 103: 528.

67. Feenstra K, vd Lee R, Greven HA, Arends A & Hoedemaeker PhJ. Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. I. The natural history: A histologic and immunohistologic study at the light microscopic and the ultrastructural level. Lab Invest 1975; 32: 235.
68. Fillit HM & Zabriskie JB. Cellular Immunity in glomerulonephritis. Am J Pathol 1982; 109, 227.
69. Fleuren GJ, Lee R vd, Greben HA, van Damme BJC & Hoedemaeker Ph.J. Experimental glomerulonephrits in the rat induced by antibodies directed against tubular antigens. IV. Investigations into the pathogenesis of the model. Lab Invest 1978; 38: 496.
70. Fleuren GJ, Grond J & Hoedemaeker PhJ. The pathogenetic role of free circulating antibody in autologous immune complex glomerulonephrits. Clin exp Immunol 1980; 41: 205.
71. Fleuren GJ, Moons WM, Slegers JFG & Hoedemaeker PhJ. In situ formation of subepithelial immune aggregates is independent of ischemia. Abstractbook 8th Int Congress of Nephrol 1981: Athens, Greece.
72. Gabbiani G, Badonnel M-C & Vassalli P. Experimental focal glomerular lesions elicited by insoluble immune complexes. Lab Invest 1975; 32: 33.
73. Geha RS. Idiotypic determinants on human T-cells. Clin Res 1980; 28: 503A.
74. Germuth EG & Rodriguez E. In: Immunopathology of the renal glomerulus. 1973. Little, Brown & Co., Boston.
75. Giorno R. Unusual structure of human splenic sinusoids revealed by monoclonal antibodies. Histochemistry 1984; 81, 505.
76. Giroux L, Verroust P, Morel-Maroger L, Delarue F, Delauche M & Srear JD.Glomerular fibrinolytic activity during nephrotoxic nephritis. Lab Invest 1979; 40: 415.
77. Glassock RJ, Edgington TS, Watson JI & Dixon FJ. Autologous immune complex nephritis induced with renal tubular antigen. II The pathogenetic mechanism. J exp Med 1968; 127: 573.
78. Graham RL, Lundholm U & Karnovsky MJ. Cytochemical demonstration of peroxidase reactivity with 3-amino-9-ethylcarbazole. J Histochem Cytochem 1965; 13: 150.
79. Gribnau FWJ. Indomethacin in experimental nephritis: studies in Heymann-type nephritis and in puromycin aminonucleoside nephropathy. Thesis, University of Nijmegen, The Netherlands, 1975.
80. Hancock WH, Kraft N, Clarke F & Atkins RC. Production of monoclonal antibodies to fibronectin, type IV collagen and other antigens of the human glomerulus. Pathology 1984; 16: 197
81. Harada K, Yamamoto T, Hara M & Kihara I. Antigenic association between kidney and thymocyte. Acta Pathol Jpn 1982; 32:483.
82. Harmon WE, Gruppe WE & Parkman R. Control of autologous immune complex nephritis. I. Suppression of the disease in the presence of T-cell sensitization. J of Immunol 1980; 124: 1034
83. Hart DMJ & Fabre JW. Major histocompatibility complex antigens in rat kidney, ureter and bladder: localization with monoclonal antibodies and demonstration of Ia-positive dendritic cells. Transplantation 1981; 31:318.
84. de Heer E, Daha MR & van Es LA. The autoimmune response in active Heymann's nephritis in Lewis rats is regulated by T-lymphocyte subsets. Cellular Immunology 1985; 92: 254.
85. de Heer E, Daha MR, Burgers J & van Es LA. Reestablishment of self tolerance by suppressor T-cells after active Heymann's nephritis. Cell Immunol 1986; 98: 28.

86. Helin H, Mustonen J, Pasternack A & Antonen J. IgM-associated glomerulonephritis. Nephron 1982; 31: 11.
87. Herbert AG & Watson JD. T-cell ontogeny: the role of a stimulator-suppressor cell. Immunology Today 1986; 7: 72.
88. Hess EV, Ashworth CT & Ziff M. Transfer of an autoimmune nephrosis in the rat by means of lymph node cells. J exp Med 1962; 115: 421.
89. Heymann W, Hackel DB, Harwood S, Wilson SGF & Hunter JLP. Production of nephrotic syndrome in rats by Freund's adjuvant and rat kidney suspensions. Proc Soc exp Biol Med 1959; 100: 660.
90. Hoedemaeker PhJ. Een vorm van experimentele immuuncomplex-glomerulonephritis. Ned T Geneesk 1972; 116: 1802
91. Hoedemaeker PhJ, Feenstra K, Nijkeuter A & Arends A. Ultrastructural localization of heterologous nephrotoxic antibody in the glomerular basement membrane of the rat. Lab Invest 1972; 26: 610.
92. Hoedemaeker PhJ, Weening JJ, Grond J, Bakker WW & Fleuren GJ. Renal antigens in experimental immune complex glomerulonephritis. In: Proc. Conference on Immunological Mechanisms in Renal Disease, Washington. 1978.
93. Hoedemaeker PhJ, Grond J & Fleuren GJ. Renal tubular antigens.Proc 8th Int Congress Nephrol Athens 1981.
94. Hoitsma AJ, van Lier HJJ, Reekers P & Koene RAP. Improved patient and graft survival after treatment of acute rejections of cadaveric renal allografts with rabbit antithymocyte globulin. Transplantation 1985; 39: 274.
95. Hsu H-C & Tsai H-M. Cyclo-Phosphamide-Induced Glomerular Injury in Newborn Mice. Lab Invest 1982; 47: 281.
96. Huang TW & Langlois JC. Podoendin. A new cell surface protein of the podocyte and endothelium. J Exp Med 1985; 162: 245.
97. Ishizaki M, Sato S, Sano J, Fukuda Y, Sugisaki Y & Masugi Y. Presence of Thy 1.1 antigen in rat glomerular mesangial cells. Biomed Res 1980; 1: 438.
98. Jaffe R, Bender BL, Santamaria M & Chung AE. Segmental Staining of the Murine Nephron by Monoclonal Antibodies Directed against the GP-2 Subunit of Laminin. Lab Invest 1984; 51: 88.
99. Jeraj K, Vernier RL, Sisson SP & Michael AF. A new glomerular antigen in passive Heymann's nephritis. Br J exp Pathol 1984; 65: 485.
100. Jerne NK. Towards a network theory of the immune system.Ann Immunol 1974; 125C: 373.
101. Jerne NK, Roland J & Cazenave P-A. Recurrent idiotopes and internal images.EMBO journal 1982; 1: 243.
102. Johnson GD & de C Nogueira Araujo GM. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 1981; 43: 349 .
103. Joling P, Tielen FJ, Vaessen LMB, Hesse CJ & Rozing J. Intrathymic differentiation in the rat. In Advances in experimental Medicine and Biology. Vol 186, edited by Klaus GGB, p 235. New York, Plenum Press, 1985.
104. Kamata K, Baird LG, Erikson ME, Collins AB & McCluskey RT. Characterization of antigens and antibody specificities involved in Heymann nephritis. J Immunol 1985, 135: 2400.
105. Kerjaschki D & Farquhar MG. The pathogenetic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. Proc Natl Acad Sci USA 1982; 79: 5557.
106. Kerjaschki D & Farquhar MG. Immunocytochemical localization of the Heymann nephritis antigen (GP 330) in glomerular epithelial cells of normal Lewis rats. J Exp Med 1983; 157: 667.

107. Kerjaschki D, Noronha-Blob L, Sacktor B & Farquhar MG. Microdomains of distinctive glycoprotein composition in the kidney proximal tubule. J Cell Biol 1984; 98: 1505.
108. Kreeftenberg, J.G. Rabbit anti-human-thymocyte globulin production, control and clinical evaluation. Academic Thesis, RIV Bilthoven, University of Amsterdam, 1979.
109. Kroese FGM, Opstelten D, Wubbena AS, Deenen GJ, Aten J, Schwander EH, Leij L de & Nieuwenhuis P. Monoclonal Antibodies to rat B-Lymphocyte (sub-)populations. Proceedings of the 8th Int Conf on Lymphatic Tissues and Germinal Centres in Immune Reactions, Cambridge 1984.
110. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680.
111. Litwin A, Adams LE, Levy R, Cline S & Hess EV. Cellular immunity in experimental glomerulonephritis of rats. I. Delayed hypersensitivity and lymphocyte stimulation studies with renal tubular antigens. Immunol 1971; 20: 755.
112. Litwin A, Adams LE, Yamauchi Y & Hess EV. Cellular immmunity in experimental glomerulonephrits of rats. II. A sequential study of immunological events occuring during development of the disease. Immunol 1973; 25: 227 .
113. Litwin A, Bash JA, Adams LE, Donovan RJ & Hess EV. Immunoregulation of Heymann's nephritis. I. Induction of suppressor cells. J Immunol 1979; 122: 1029 .
114. Madaio MP, Salant DJ, Cohen AJ, Adler S & Couser WG. Comparative study of in situ immune deposit formation in active and passive Heymann nephritis. Kidney Int 1983; 23: 498.
115. Makker SP. Brush border antibodies of Heymann nephritis of rats in normal human serum. Proc Soc Exp Biol and Med 1977; 154: 9.
116. Mason DW & Williams AF. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. Biochem J 1980; 187: 1.
117. Matsuo S, Caldwell PRB, Brentjens JR & G Andres. In vivo interaction of antibodies with cell surface antigens. A mechanism responsible for in situ formation of immune deposits in the zona pellucida of rabbit oocytes. J Clin Invest 1985; 75: 1369.
118. Mendrick DL, Rennke HG & Cotran RS, Springer TA, Abbas AK. Methods in Laboratory Investigation Monoclonal Antibodies against Rat Glomerular Antigens: Production and Specificity. Lab Invest 1983; 49: 107.
119. Miettinen A, Tornroth T, Ekblom P, Virtanen I & Lindner E. Nephritogenic and non-nephritogenic epithelial antigens in autoimmune and passive Heymann nephritis. Lab Invest 1984; 50: 435.
120. Monaco AP, Campion J-P & Kapnick SJ. Anti-lymphocyte globulins: Clinical use of anti-lymphocyte globulin. Transpl Proc 1977; 9: 1007.
121. Morita T, Kihara I, Oite T, Yamamoto T & Suzuki Y. Mesangiolysis Sequential Ultrastructural Study of Habu Venom-Induced Glomerular Lesions. Lab Invest 1978; 38: 94.
122. Morita T & Churg J. Mesangiolysis: Editorial Review. Kidney Int 1983; 24: 1.
123. Morris RJ & Ritter MA. Association of Thy-1 Cell Surface Differentiation Antigen with Certain Connective Tissues in vivo. Cell Tissue Res 1980; 206: 459.
124. Muller, E. (personal communication).
125. Naparstek Y, Holoshitz J, Eisenstein S, Reshef T, Rappaport S, Chemke J, Ben-Nun A & Cohen JR. Effector T-lymphocyte line cells migrate to the thymus and persist there. Nature 1982; 300:262.

126. Naruse T, Kitamara K, Miyakawa Y & Shibata S. Deposition of renal tubular epithelial antigen along the glomerular capillary walls of patients with membranous glomerulonephritis. J Immunol 1973; 110: 1163.
127. Naruse T, Fukasawa T & Miyakawa Y. Laboratory model of membranous glomerulonephritis in rats induced by pronase-digested homologous renal tubular epithelial antigen. Lab Invest 1975; 33: 141.
128. Naruse T, Fukasawa T, Hirokawa N, Oike S & Miyakawa Y. The pathogenesis of experimental membranous glomerulonephrits induced with homologous nephritogenic tubular antigen. J Exp Med 1976; 144: 1347.
129. Naysmith JD, Elson CJ, Dallman M, Fletcher E & Ortega-Pieres MG. Anti-erythrocyte autoantibody production in mice associated with the injection of rat erythrocytes. I. Regulation of the response by suppressor cells. Immunol 1980; 39: 469.
130. Neale TJ & and Wilson CB. Fixed glomerular antigen in Heyman's nephritis: eluted antibody reactivity with normal rat glomerulus. Kidney Int. 1979; 16: 799.
131. Neale J & Wilson CB. Glomerular antigens in Heymann's nephritis: reactivity of eluted and circulating antibody. J Immunol 1982; 128: 323.
132. Neilson EG & Phillips SM. Suppression of interstitial nephritis by auto-anti-idiotypic immunity. J Exp Med 1982; 155: 179.
133. Neilson EG & Zakheim B. T cell regulation, anti-idiotypic immunity, and the nephritogenic immune response. Kidney Int 1983; 24: 9.
134. Nisonoff A & Lamoyi E. Implications of the presence of an internal image of the antigen in anti-idiotypic antibodies: possible application to vaccine production. Clin Immunol and Immunopathol 1981; 21: 397.
135. Okumura K, Tada T & Ochiai T. Effect of anti- thymocyte serum on reaginic antibody formation in the rat. Immunol 1974; 26:256.
136. Paul LC, Rennke HG, Milford EL & Carpenter CB. Thy 1.1 in glomeruli of rat kidneys.. Kidney Int 1984; 25: 771.
137. Perkinson DT, Baker PJ, Couser WG, Johnson RJ & Adler S. Membrane Attack Complex Deposition in Experimental Glomerular Injury. Am J Pathol 1985; 120: 121.
138. Platt JL, LeBien TW & Michael AF. Stages of renal ontogenesis identified by monoclonal antibodies reactive with lymphohemopoietic differentiation antigens. J Exp Med 1983; 157: 155.
139. Ronco P, Allegri L, Melcion C, Pirotsky E, Appay M-C, Bariety J, Pontillon F & Verroust P. A monoclonal antibody to brush border and passive Heymann nephritis. Clin exp Immunol 1984; 55: 319.
140. Ronco P, Melcion C, Geniteau M, Ronco E, Reininger L, Galceran M & Verroust P. Production and characterization of monoclonal antibodies against rat brush border antigens of the proximal convoluted tubule. Immunology 1984; 53: 87.
141. Ronco P, Baudoin B, Brunisholz M & Verroust P. In vivo localization of monoclonal anti GP 330 antibodies: heterogeneity of the antigen antibody system. Kidney Int 1985; 27: 221.
142. Ronco P, Neale TJ, Wilson CB, Galceran M & Verroust P. An immunopathologic study of a 330-kD protein defined by monoclonal antibodies and reactive with anti-RTEa5 antibodies and kidney eluates from active Heymann nephritis. J Immunology 1986; 136: 125.
143. Rozing J, Vaessen LMB, Faber L, van Oven M, de Vries-Box L, de Jong B & Nieuwenhuis P. Pre B cell leukaemia in the rat. In: Nieuwenhuis P, van den Broek AA, Hanna Jr MG, eds. Advances in experimental medicine and biology; 149: In vivo immunology. Plenum Press: New York, 1982: 111.

144. Rozing J, Joling P & Vaessen LMB. Determinants on the Thy-1 molecule expressed differentially during lympho-hematopoietic differentiation. Abstract book of the Fifth International Workshop of Alloantigenic Systems in the Rat, Montreal 1984.
145. Sakaguchi S, Takahashi T & Nishizuka Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. J Exp Med 1982; 156: 1577.
146. Salant DJ, Belok S, Stilmant MM, Darby C & Couser WG. Determinants of glomerular localization of subepithelial deposits. Effects of altered antigen to antibody ratio, steroids, vasoactive amine antagonists, and aminonucleoside of puromycin on passive Heymann nephritis in rats. Lab Invest 1979; 41: 89.
147. Salant DJ, Darby C & Couser WG. Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. J Clin Invest 1980; 66: 71.
148. Salant DJ, Madaio MP, Adler S, Stilmant MM & Couser WG. Altered glomerular permeability induced by F(ab')2 and Fab' antibodies to rat renal tubular epithelial antigen. Kidney Int 1981; 21: 36.
149. Scheinman JI, Fish AJ, Brown DM & Michael AJ. Human Glomerular Smooth Muscle (Mesangial) Cells in Culture. Lab Invest 1976; 34: 150.
150. Schneeberger EE, Leber PD, Karnovsky MJ, & McCluskey RT. Altered functional properties of the renal glomerulus in autologous immune complex nephritis. An ultrastructural tracer study. J Exp Med 1974, 139, 1283.
151. Schreiner GF, Kiely JM, Cotran RS & Unanue ER. Characterization of resident glomerular cells in the rat expressing Ia determinants and manifesting genetically restricted interaction with lymphocytes. J Clin Invest 1981; 68: 920.
152. Searle J, Kerr JFR & Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annual 1982; 17: 229.
153. Seelig HP, Seelig R & Roth E. Antibodies reacting with the glomerular mesangium. Virchows Arch. (Pathol. Anat) 1975; 366: 313.
154. Sege K & Peterson PA. Use of anti-idiotypic antibodies as cell-surface receptor probes. Proc Natl Acad Sci USA 1978; 75: 2443.
155. Shigematsu H, Niwa Y, Takizawa J & Akikusa B.Arhustype nephritis I. Characterization of glomerular lesions induced by insoluble and poorly soluble immune complexes. Lab. Invest 1979; 40: 492.
156. Simmons RT, Moberg AW, Gewurz H, Soll R, Tallent MB & Najarian JS. Immunosuppresive assay of anti-lymphoblast globulin in man: effect of dose, histocompatibility and serologic response to horse gamma globulin. Surgery 1970; 68: 62.
157. Slegers JFG & Foerster MTG. Absence of effect of diazoxide on sodium and water handling in the isolated perfused rat kidney. Archs int Pharmacodyn Ther 1980; 244: 309.
158. Starzl TE, Porter KA, Iwasaki Y, Marchioro TL & Kashiwagi N. The use of heterologous anti-lymphocyte globulin in human renal homotransplantation. In: Anti-lymphocytic serum, p. 4 (Wolstenholme GEW and M O'Connor eds.)1967. Churchill Ltd, London WT.
159. Stenglein, B, Thoenes, GH & Gunther E. Genetically controlled autologous immune complex glomerulonephritis in rats. J Immunol 1975; 115: 895,
160. Stenglein B, Thoenes GH & Gunther E. Genetic control of susceptibility to autologous immune complex glomerulonephritis in inbred rat strains. Clin exp Immunol 1978; 33: 88.

161. Strom B, Tilney NL, Merrill JP. Renal transplantation: clinical management of the transplant recipient. In: Brenner BM, Rector FC, eds. The kidney. Philadelphia: Saunders, 1981: 2618.
162. Sy M-S, Dietz MH, Germain RM, Benacerraf B & Greene MI. Antigen- and receptor-driven regulatory mechanisms. IV. Idiotype bearing I-J+ suppressor T cell factors induce second order suppressor T cells which express anti-idiotypic receptors. J Exp Med 1980; 151: 1183.
163. Takamiya H, Batsford, S & Vogt, A(1984) Abstractbook 9th International Congress of Nephrology, 263 A Los Angeles.
164. Tasiaux N, Leuwenkroon R, Bruyns C & Urbain J. Possible occurence and meaning of lymphocytes bearing auto-anti-idiotypic receptors during the immune response. Eur J Immunol 1978; 8: 464,
165. Tauc M, Poujeol P, Verroust P, Ronco P, Brianti P, Chatelet F & van de Walle A. Characterization of renal brushborder (BB) aminopeptidase by a monoclonal antibody. Kidney Int 1985; 27: 276.
166. Terasaki P, Cats S, Toyotome A, Iwaki Y, Kinukawa T, Wakisaka A, Takahashi H, Hardiwidjaja & Mendez R. Use of monoclonal antibodies for kidney transplant patients. Transplant Proc 1985; 17: 1521.
167. Thiel G, Moppert F, Gudat J, Zollinger HU, Brunner F, Enderlin F & Harder F. Follow-up of glomerular changes in man after intravenous administration of anti-lymphocyte globulin containing anti-glomerular basement membrane antibodies (anti-GBM-ab). In: ALG therapy and standardization workshop, p. 242 (Seiler FR & HG Schwick eds.)1972. Behringwerke AG, Marburg.
168. Thomas WR, Morahan G & Miller JFAP. Induction of suppressor T-cells by monoclonal anti-idiotope antibody in strains of mice not expressing the idiotope in hyperimmune serum. J Immunol 1983; 130: 2079.
169. Timens W & Poppema S. Lymphocyte compartments in human spleen. An immunohistologic study in normal spleens and noninvolved spleens in Hodgkin's Disease. Am J Pathol 1985; 120: 443.
170. Towbin H, Staehelin T & Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350.
171. Unanue ER, Beller DI, Lu CY & Allen PM. Antigen presentation: comments on its regulation and mechanism. J Immunol 1984; 132: 1,
172. Urbain J & Wuilmart C. Some thoughts on idiotypic networks and immunoregulation. Immunology Today 1982; 3: 88.
173. Veassen LMB, Joling P, Tielen FJ & Rozing J. New surface antigens on cells in the early phase of T-cell differentiation, detected by monoclonal antibodies. In Advances in experimental Medicine and Biology. Vol 186, edited by Klaus GGB, p 251. New York, Plenum Press, 1985.
174. Whitworth JA, Leibowitz S, Kennedy MC, Cameron JS, Evans DJ, Glassock RJ & Schoenfeld LS. Absence of glomerular renal tubular epithelial antigen in membranous glomerulonephritis. Clin Nephrol 1976; 5: 159.
175. Wiggins RC, Glatfelter A & Brukman J. Procoagulant activity in glomeruli and urine of rabbits with nephrotoxic nephritis. Lab Invest 1985; 53: 156.
176. Williams AF, Barclay AN, Letharte-Muirhead M & Morris RJ. Rat Thy-1 antigens from thymus and brain: Their tissue distribution purification and chemical composition. Cold Spring Harbor Symp Quant Biol 1977; 41: 51.
177. Wilson CB & Dixon FJ. The Kidney, edited by Brenner BM, Rector FC. Philadelphia, Saunders WB, 1981, pp 1237, Chapter: The renal response to immunologic injury.

178. Woodroffe AJ & Wilson CG. An evaluation of elution techniques in the study of immune complex glomerulonephritis. J Immunol 1977; 118: 1788.
179. Yamamoto T & Wilson CB. Antibody induced mesangial cell damage: the model, functional alterations, and effects of complement. Kidney Int 1986; 29: 296 (abstract) .
180. Zanetti M, Barton RW & Bigazzi PE. Anti-idiotypic immunity and auto-immunity. II. Idiotypic determinants and auto-antibodies and lymphocytes in spontaneous and experimentally induced auto-immune thyroiditis. Cell Immunol 1983; 75: 292.